

MORPHOLOGICAL AND ULTRASTRUCTURAL STUDIES ON SPORES  
AND GERM TUBES OF SELECTED ARBUSCULAR MYCORRHIZAL FUNGI  
(GLOMALES)

By

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Abstract of Dissertation Presented to the Graduate School  
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MORPHOLOGICAL AND ULTRASTRUCTURAL STUDIES ON SPORES  
AND GERM TUBES OF SELECTED ARBUSCULAR MYCORRHIZAL FUNGI  
(GLOMALES)

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Morphological and ultrastructural studies were conducted to clarify the nature of the spores formed in Glomales, an order of arbuscular mycorrhizal fungi. As the first step, techniques for fixation and embedding of these spores were tested. Of nine methods none was effective for fixation of *Acaulospora*, *Gigaspora* and *Scutellospora* spores, but one was efficient for fixation of *Glomus*. This method included fixation with glutaraldehyde in a microwave oven, and breaking the spores under liquid N<sub>2</sub> before postfixation with osmium tetroxide. For spore walls, however, most of the techniques provided reasonable results in all of the genera tested.

It was observed that laminated walls are formed by different developmental processes. A wall with apparent

arcuate distribution of fibrils at the ultrastructural level is common in all genera studied, but it does not always correspond to a laminated wall observed with the LM, nor do all laminated walls have this pattern of fibrillar distribution. Special features reported to occur in walls of other Glomales species, such as a perpendicular periodic layer of fibrils and a striate layer, were confirmed here in other species, indicating that these characters as well as apparent arcuate fibrils are unique to Glomales. Outer, evanescent walls are regularly degraded by bacteria. However, the bacteria apparently are not able to degrade outer, persistent walls, even when they live on them.

A different method of germination with formation of a special chamber for germ tube initiation was demonstrated in *Gigaspora albida*, and it seems to be an intermediary form between the germination processes of *Gigaspora margarita* and *Scutellospora* species. No clear proof of meiotic or mitotic processes were found, but some evidence, such as elongated, paired and linked nuclei, presence of nucleus-associated organelles and a line of division in the central region of some of the nuclei, demonstrated that nuclear condition changes in spores and germ tubes. The sexual nature of the spores in Glomales could not be established. More studies on cytology and development are necessary to establish if they represent anamorphic or teleomorphic states.

## CHAPTER 1 GENERAL INTRODUCTION

Mycorrhiza is a mutualistic symbiosis where a fungus and its host (plant roots) co-exist, as defined by Harley (1989), "...in a physiologically, ecologically, and reproductively active stage for long periods." In fact, most of the mycorrhizal fungi are perennial and may persist for extremely long periods (Trappe and Molina, 1986). The most common type of mycorrhiza is the arbuscular, which is characterized by formation of arbuscules in the root cortex. The arbuscular mycorrhizal fungi are obligate biotrophs which play an important role in natural and agricultural agroecosystems by enhancing plant growth (Hayman and Tavares, 1985). They form mycorrhizal association with a wide variety of hosts, from Bryophytes and Pteridophytes to Gymnosperms and Angiosperms (Harley, 1989). The evolution of concepts about the arbuscular mycorrhizal fungi have demonstrated that their important role is related not only with the plant host but with all the surrounding environment. Thus, they constitute a key component of the rhizosphere ecosystem (Linderman, 1988, 1991). Bethlenfalvay and Newton (1991) stress this point, arguing that arbuscular mycorrhizal fungi should be considered not

only as a plant symbiont but also as a vital interface connecting plant and soil communities.

All fungi forming arbuscular mycorrhizal associations are classified among the Zygomycetes and were for many years included in the Endogonaceae (Mucorales). Thaxter (1922) considered the family Endogonaceae to consist of four genera: *Endogone* Link, *Sclerocystis* Berkeley & Broome, *Sphaerocreas* Saccardo & Ellis, and *Glaziella* Berkeley. *Sphaerocreas* was later transferred to *Endogone* (Zycha, 1935). Moreau (1953) proposed the order Endogonales to separate the Endogonaceae from Mucorales but that was validated only when Benjamin (1979) added its Latin diagnosis. In a revision of Endogonaceae, Gerdemann and Trappe (1974) divided *Endogone* in four genera: *Endogone* sensu stricto, *Glomus* Tulasne & Tulasne, *Gigaspora* Gerd. & Trappe emend. Walker & Sanders and *Modicella* Kanouse. In addition, they described a new genus, *Acaulospora* Gerd. & Trappe and also considered the genera *Sclerocystis* and *Glaziella* as components of the family. Subsequently, three new genera were included in the Endogonaceae: *Entrophospora* Ames & Schneider (1979), *Complexipes* Walker (1979) and *Scutellospora* Walker & Sanders (1986). *Modicella* was transferred to Mortierellaceae (Trappe and Schenck, 1982), while *Complexipes* (Danielson, 1982) and *Glaziella* (Gibson et al., 1986) were shown to be Ascomycetes. Pirozynski and Dalpé (1989) proposed the family Glomaceae to include *Glomus*

and *Sclerocystis* based on fossil records where specimens similar to extant species of these genera were found.

Six of the seven genera that remained in Endogonales were recently moved to a new order, Glomales (Morton and Benny, 1990). Exclusion of these genera from Endogonales was mainly based on habit. As proposed by Morton and Benny (1990), Glomales includes "... all soil borne fungi which form arbuscules in obligate mutualistic associations with terrestrial plants." Endogonales was maintained with a single genus, *Endogone*, which is a typical saprophyte but has occasionally been found forming ectomycorrhizal associations (Fassi et al., 1969). The genera transferred to Glomales were distributed in three families: Glomaceae, with the genera *Glomus* and *Sclerocystis*, as proposed by Pirozynski and Dalpé (1989); Acaulosporaceae including the genera *Acaulospora* Gerdemann & Trappe emend. Berch and *Entrophospora*; and Gigasporaceae with two genera: *Gigaspora*, and *Scutellospora*. The families Glomaceae and Acaulosporaceae, whose members form chlamydospores and produce arbuscules and vesicles in the associated host, were included in a suborder, Glomineae. The family Gigasporaceae was placed in the suborder Gigasporineae. Members of this family form spores considered to be azygospores, extraradical auxiliary cells, and no internal vesicles (Morton and Benny, 1990).

The order Glomales is now a broad group, with almost a hundred and fifty species. The genus with the most described species is *Glomus*, followed by *Scutellospora*, *Acaulospora*, *Gigaspora*, *Entrophospora*, and *Sclerocystis* (Almeida, 1989; Almeida and Schenck, 1990; Morton, 1988). Recently the genus *Sclerocystis* was revised by Almeida and Schenck (1990), who proposed the maintenance of this genus with only one species, *Sclerocystis coremioides* Berk. & Broome. The other genera were considered as synonymous or were moved to the genus *Glomus*.

Evolutionary relationships among Zygomycetes, especially Glomales, is not clear. The general characters of the class are non-septate mycelium, asexual reproduction by formation of endospores, and sexual reproduction by zygospores. Arbuscular mycorrhizal fungi, as obligate symbionts, do not grow in pure culture and this has tremendously limited the study of the group. Thus, in Glomales the true nature of the spores is still uncertain. Their sexual stage has been described but poorly documented for only one species (Tommerup, 1988; Tommerup and Sivasithamparam, 1990). The other spores formed by members of this order have been considered as azygospores, chlamydospores (Gerdemann and Trappe, 1974; Morton, 1988; Morton and Benny, 1990; Schenck and Pérez, 1990; Siqueira et al., 1985), conidia (Tommerup and Sivasithamparam, 1990), or sporangiospores (Gibson, 1985; Walker, 1987), although there

are no descriptions on cytological formation of such spores. Consequently, their life cycles are imperfectly understood (Walker, 1985).

Species of the genera *Gigaspora* and *Scutellospora* form spores on bulbous suspensor-like cells, which resemble the suspensors formed during sexual reproduction of other Zygomycetes. For this reason these spores were considered as azygospores formed on parthenogenetic gametangia (Gerdemann and Trappe, 1974). A different and smaller spore formed by one species of *Gigaspora* was described as a zygosporangium by Tommerup (1988). These observations were based largely on morphological features.

Species of *Acaulospora* and *Entrophospora* produce spores respectively within a lateral inflation or completely within the neck of a swollen hyphal terminus (Walker, 1987). These spores are mentioned as azygospores (Schenck and Pérez, 1990) since the terminal hypha resembles an aborted gametangium, and they have also been considered as chlamydospores (Morton and Benny, 1990) or as single-spored sporangia (Gibson, 1985; Walker, 1987).

Representatives of *Glomus* and *Sclerocystis* produce spores borne terminally or intercalarily on one or more hyphae and are considered to be chlamydospores (Gerdemann and Trappe, 1974; Morton and Benny, 1990; Schenck and Pérez, 1990) or conidia (Tommerup and Sivasithamparan, 1990).

The arbuscular mycorrhizal fungi form an extra- and an intra-matrical phase. The former is represented by mycelia, spores, and auxiliary cells; the last, more complex, consists of hyphal coils, intercellular hypha, inter- and intracellular vesicles and arbuscules (Bonfante-Fasolo, 1987; Sylvia, 1990). Colonization within the root has been studied in detail (Bonfante-Fasolo, 1986, 1987; Carling and Brown, 1982; Cook et al. 1988; Smith and Walker, 1981), using both the light microscope (Abbott, 1982; Abbott and Robson, 1979; Sward, 1978), and electron microscopy (Bonfante-Fasolo and Scannerini, 1977; Cox and Sanders, 1974; Gianinazzi-Pearson et al. 1981). Scannerini and Bonfante-Fasolo (1983) reviewed the literature on ultra-structural analysis of mycorrhizal associations. Later, Bonfante-Fasolo (1986) reviewed the same subject, describing the extra- and intramatrical phases and specifying how mycorrhizal colonization develops in Bryophytes, Pteridophytes, Gymnosperms, and Angiosperms. Gianninazzi-Pearson and Gianinazzi (1988) reviewed the literature related with morphological integration and compatibility functional between the partners in the arbuscular mycorrhizal symbiosis. Most of the work that has been done emphasizes the root fungi interaction (Abbott, 1982; Bonfante-Fasolo, 1978; Bonfante-Fasolo and Scannerini, 1977; Cox and Sanders, 1974; Gianinazzi-Pearson et al., 1981; Kariya and Toth, 1981), with a few reports (Gibson, 1985; Mosse, 1970a,

1970b, 1970c; Old et al., 1973; Sward, 1981a, 1981b, 1981c) on the ultrastructure of the fungi. Mosse (1970c) and Sward (1981b) indicated that the species of arbuscular mycorrhizal fungi they studied were related to the Mucorales based on similarities in wall formation, described as "de novo" formation of a vegetative wall under the spore wall by Bartnicki-Garcia (1968), and for the presence of chitin in their walls. Gibson (1985) found indications of close affinity between the genera he studied, *Endogone*, *Glomus*, *Gigaspora* and *Sclerocystis*, emphasizing the similarities in staining properties between the outer wall of *Gigaspora* and *Glomus* species with zygosporangial walls of *Endogone*, and the presence of multiperforate septa in *Sclerocystis*, *Glomus*, and *Endogone*. He considered the perforate septa as a familial character linking these three genera.

The most important characters used in separating the genera and species of Glomales are, respectively, spore ontogeny, modes of spore germination (Morton and Benny, 1990; Siqueira et al. 1985), and wall characteristics (Morton and Benny, 1990; Walker, 1983). It has been considered that details of nuclear division, septation, and spore development may have phylogenetic importance. Knowledge of these features constitutes a critical source of information for evaluating taxonomy and evolution (O'Donnell and McLaughlin, 1984). However, since Cutter's work (1942) it has been shown how difficult and, many times, deceptive are

the studies regarding nuclear behavior in different groups of fungi. It is interesting that most of the reasons pointed out by Cutter (1942) at that time are still valid today and have seriously contributed to the lack of clear understanding of reproductive events and nuclear behavior in the Zygomycetes. Among those are: use of unsatisfactory cytological techniques, the high natural variation of material used in the studies, the failure of many workers to follow the nuclear behavior through all stages of the life cycle, and especially the extremely minute nature of mucoraceous nuclei (Cutter, 1942). With respect to arbuscular mycorrhizal fungi there are no conclusive studies of their biochemical, physiological or genetic properties, nor do we know of any cytological or ultrastructural description of their processes of reproduction. As pointed out by Harley (1986), we do not know about their nuclear or sexual behavior or potential gene flow. The application of transmission electron microscopy (TEM), although with its own limitations, has greatly increased the knowledge of nuclear structure in fungi (Beakes, 1981). Therefore, since the true nature of the arbuscular mycorrhizal spores is not clear, it becomes evident that more detailed cytological and ultrastructural studies are needed in order to better understand this group of fungi.

The general objective of this research is to determine the systematic relationship of the Endogonales and Glomales

using morphological, cytological, and ultrastructural studies. In order to achieve specific objectives, attempts were made to: a) locate and study nuclear phenomena; b) examine organelle changes during spore germination; c) study spore formation; and d) add new information about wall ultrastructure. However, the arbuscular mycorrhizal fungi proved to be extremely difficult to study, especially because of problems with fixation and embedding of spores and germ tubes. Since these fungi do not grow in axenic culture and must be maintained in association with a living host, it is difficult to "clock" the events of the life cycle and to find material at specific times which would allow the study of each phase of the cycle.

In this work, a general view of ultrastructural studies with some species of arbuscular mycorrhizal fungi is given in Chapter I. In Chapter II, the major problems related to fixation of arbuscular mycorrhizal structures and a technique that proved to be useful for fixation of *Glomus* spores will be described. Since spore walls have been widely accepted as a taxonomic character for differentiation of arbuscular mycorrhizal species, it was considered important to study them at the ultrastructural level, in order to understand and better clarify certain features observed with the light microscope. Chapter III discusses spore wall formation and structure in different representatives of the arbuscular mycorrhizal group and

bacterial colonization of these walls. There were observed significant changes in distribution of organelles from mature spores to germ tubes. Meiotic events were not discovered, but some stages characteristics of mitosis were recognized. This subject is dealt with in Chapter IV. A general summary and conclusions of these investigations will be presented in Chapter V.

CHAPTER II  
PROBLEMS WITH FIXATION AND EMBEDDING  
OF ARBUSCULAR MYCORRHIZAL FUNGI

Introduction

One of the greatest problems in electron microscopy is often in the preparation of material for examination. In general the processes of fixation and embedding are time consuming and require special techniques, chosen according to the material that is being studied. With arbuscular mycorrhizal fungi the difficulties are increased due to the nature of membranes and thickness of spore walls which represent a barrier for penetration of fixatives, such as dilute aldehydes and osmium tetroxide, and embedding mixtures. Consequently, ultrastructural studies of spores of arbuscular mycorrhizal fungi have been restricted largely to spore wall structure (Bonfante-Fasolo, 1982; Bonfante-Fasolo and Schubert, 1987; Bonfante-Fasolo and Vian, 1984; Bonfante-Fasolo et al., 1985, 1990; Gibson et al., 1987; Grippiolo and Bonfante-Fasolo, 1984; Old et al., 1973), with limited information on cytological events and organelles within spores and hyphae. Exceptions are the investigations of Mosse (1970a, 1970b, 1970c), Sward (1981a, 1981b, 1981c),

and Gibson (1985) which have provided the most representative and detailed studies on spores of arbuscular mycorrhizal species at the ultrastructural level. Mosse (1970b) used 3% acrolein in 0.025 M cacodylate buffer (pH 7.1), followed by 1% osmium tetroxide, in her study of honey-colored, sessile *Endogone* spores, later recognized as *Acaulospora laevis* Gerd. & Trappe. In this case, for better penetration of the epoxy-resin (araldite), it was necessary to pierce the spores with a fine needle. Mosse (1970b) also mentioned that by using glutaraldehyde, glutaraldehyde plus formalin, and osmium only, the results were the same or worse than with acrolein. Sward (1981a) fixed spores of *Gigaspora margarita* Becker & Hall in a mixture of 3% (v/v) glutaraldehyde and 3% (v/v) acrolein in 0.1 M Na-Na<sub>2</sub> phosphate buffer at pH 7.2. Gibson (1985) also utilized acrolein (1.5%), with 1.5% paraformaldehyde and 3% glutaraldehyde for fixation of spores of *Acaulospora*, *Gigaspora*, *Glomus*, and *Scutellospora* species. Good preservation of walls was obtained in all genera. However, effectiveness of fixation and embedding of sporoplasm varied according to the genera and species examined. Spore contents of *Gi. margarita* and *Gi. gigantea* (Nicol. & Gerd.) Gerd. & Trappe were the only ones well preserved; in *Scutellospora gregaria* (Schenck & Nicol.) Walker & Sanders only the wall was structurally distinct and in *S. heterogama* (Nicol. & Gerd.) Walker & Sanders and *S. pellucida* (Nicol. & Schenck)

Walker & Sanders sporoplasm structures were not well fixed and embedded. With species of *Glomus* results were poor, except for some walls, with sporoplasm not distinguishable at the electron microscope level. *Glomus intraradices* Schenck & Smith was examined also in association with the root and, in this case, fixation of intraradical structures (arbuscules and hyphae) was better than fixation of spores.

There are many ultrastructural studies of the endomycorrhizal association. As considered by Scannerini and Bonfante-Fasolo (1983), studies with the TEM make it possible to understand the cell wall interactions in the mutualistic symbiotic state. In the same way, fixation and embedding of the colonized root provide an alternative for studying the arbuscular fungi. In the host these fungi can be found in the epidermis, hypodermis, and root cortex, but they never colonize the central cylinder. Traditional fixation with glutaraldehyde and osmium tetroxide (Bonfante-Fasolo, 1982; Bonfante-Fasolo and Grippiolo, 1982; Bonfante-Fasolo and Vian, 1989; Bonfante-Fasolo et al., 1990; Codignola et al., 1989; Garriock et al. 1989; Kreutz-Jeanmaire et al., 1988), glutaraldehyde plus paraformaldehyde (Yawney and Schultz, 1990), and glutaraldehyde plus paraformaldehyde plus dimethyl sulfoxide (Jabaji-Hare et al., 1990) have been employed for these studies. A general review and a comparative ultrastructural analysis of diverse mycorrhizal associations were provided

by Scannerini and Bonfante-Fasolo (1983); they concluded that the arbuscular mycorrhizae are the most complex group of mycorrhizal fungi, at least when the intraradical phase is considered. The anatomy of an arbuscular mycorrhizal symbiosis was described in studies with sugar maple (*Acer saccharum* Marsh) and *Glomus etunicatum* Becker & Gerdemann (Yawney and Schultz, 1990). Among other conclusions, the authors confirmed that the arbuscule is the site of transfer between fungus and plant root. The ultrastructural organization of the arbuscular mycorrhizae formed between *Gl. intraradices* and two species of Rosaceae was described by Kreutz-Jeanmaire et al. (1988). In this work the authors investigated especially the structural changes in the host cell during the development of the arbuscules by the fungus. Bonfante-Fasolo and Vian (1984) described morphological and cytochemical changes of wall components from the extraradical to the intraradical phase of *Gl. fasciculatum* (Thaxter) Gerd. & Trappe emend. Walker & Koske, associated with *Vitis vinifera* L. Using conventional fixation for TEM and cryoultramicrotomy, Bonfante-Fasolo (1982) observed the cell wall architectures of both the fungus and the host in a mycorrhizal association between *Ornithogalum umbellatum* L. and *Gl. fasciculatum*. The ultrastructural texture of cell walls of *Allium porrum* L. roots associated or not associated with *Glomus versiforme* (Karst.) Berch (syn. *Gl. epigaeum* Daniels & Trappe) was compared by Bonfante-Fasolo and Vian

(1989), while the early stages of colonization in this system were described by Garriock et al. (1989). The same mycorrhizal association was used to show the correlation between chitin distribution and cell wall morphology of the fungus (Bonfante-Fasolo et al., 1990). Codignola et al. (1989) studied the chemical composition of phenols and their localization in the cell wall of arbuscular mycorrhizal and non-mycorrhizal roots of *Allium porrum* and *Ginkgo biloba* L. They have shown that the constitutive phenols in the cell wall do not change qualitatively or quantitatively after colonization of roots by the arbuscular mycorrhizal fungus. In roots of leek (*Allium porrum*) colonized or not by *Glomus clarum* Nicol. & Schenck, the presence of different sugars was ultrastructurally examined using lectin-gold complexes (Jabaji-Hare et al., 1990). Results indicated that production of some sugars in the host had been triggered during the association. The authors were also able to identify various cell wall and cytoplasmic carbohydrates in the fungus and in the host tissue. The use of ultrastructural and affinity techniques, such as enzyme-gold labelling, immunogold labelling, and dot-blot immunoassay, allowed the study of the interface formed between *Gl. versiforme* and *Allium porrum* (Bonfante-Fasolo et al., 1990), and a better understanding of this host-fungus interaction. The colonization of tomato roots by a virus and by an arbuscular mycorrhizal fungi, *Glomus* sp. has also been the

object of ultrastructural studies (Jabaji-Hare and Stobbs, 1984). Although these studies strongly contribute to a better understanding of the arbuscular mycorrhizal symbiosis, it is evident that use of fixed roots does not provide enough information about the mycorrhizal fungus itself, since only hyphae, arbuscules and vesicles have been observed.

Studies of arbuscular mycorrhizal fungi at the light microscope level are in the same way limited and cannot provide enough information for understanding the processes of formation, development, and germination of arbuscular mycorrhizal spores. Thus, efforts were made to develop techniques which could be regularly used for fixation and embedding of such spores. The use of acrolein was avoided since it has already been used without satisfactory results, especially for spores of *Glomus* species.

#### Materials and methods

##### Isolates Tested

Isolates of different arbuscular mycorrhizal genera were used in order to evaluate methods that would be most suitable for fixation of their spores. All isolates were originally deposited at the International Culture Collection of VA Mycorrhizal Fungi (INVAM). Scientific names are spelled as suggested by Almeida (1989), with the exception

of *Scutellospora*, which is maintained as originally described (Walker and Sanders, 1986; Walker, 1991).

*Acaulospora longula* Spain & Schenck (INVAM - ALGL 372)

*Acaulospora scrobiculata* Trappe (INVAM - ASCB 984)

*Acaulospora morrowiae* (INVAM - AMRW 983)

*Glomus claroides* (INVAM - LCRD 698)

*Glomus* sp. (INVAM - L...906)

*Glomus intraradices* Schenck & Smith (INVAM - LITR 208)

*Glomus* sp. (INVAM - L...312)

*Glomus* sp. (INVAM - L...925)

*Gigaspora margarita* Becker & Hall (INVAM - GMRG 144)

*Gigaspora albida* Schenck & Smith (INVAM - GABD 927)

*Scutellospora* sp. (INVAM - C... 339)

*Scutellospora heterogama* (Nicol. & Gerd.) Walker & Sanders (INVAM - CHTG 400).

### Cultural Procedures

These isolates were pot-cultured in a soilless medium which consisted of a mixture of 3:1 (v/v) sand and vermiculite (Liyanage, 1989), in association with Bahia grass (*Paspalum notatum* Flugge). The pot cultures received Long Ashton nutrient solution (Hewitt, 1966) every other day and were maintained in a growth room with artificial mercury light (400 to 600  $\mu\text{mol. cm}^{-2} \text{ sec}^{-1}$ ) of 14 hr length. Temperature ranged between 26°C and 28°C (day/night).

*Glomus* sp. (isolate 906) was also maintained in aeroponic culture (Hung and Sylvia, 1988), associated with sweet potato (*Ipomoea batatas* (L.) Lam., cultivar white star). Colonized seedlings were placed into aeroponic chambers, which contained diluted Hoagland nutrient solution, as described in Sylvia and Hubbell (1986). The solution was changed every fifteen days. Spores were collected at different time intervals and separated from the roots to which they were attached, or from root debris in the water, with needles and Eppendorf pipettes, under a dissecting microscope.

Spores were isolated from pot cultures by wet sieving and decanting (Gerdemann and Nicolson, 1963), followed by centrifugation in 40% (v/v) sucrose (Jenkins, 1964).

After isolation in Petri dishes, a high number of spores (minimum of 500 for species of *Glomus* and *Acaulospora*; minimum of 200 for species of *Gigaspora* and *Scutellospora*) was selected, using an Eppendorf pipette, and transferred to a small plastic or glass container. In a few cases when the amount of available spores was small, they were placed in 2% agarose. When solidified, narrow strips of agarose containing 1-2 spores were cut with a razor blade and processed for fixation. Different methods for fixation were tried, as described below. In all cases spores which were not in agar blocks, were centrifuged (1500 X g/30 sec)

between each fixation and embedding step, which allowed the change of solutions with small loss of material.

#### Fixation for EM

Eight methods for fixation of spores were attempted. Detailed descriptions of these techniques are given below.

a) Spores were fixed in 7.2% glutaraldehyde for two hours at room temperature or overnight at 5°C, rinsed three times in 0.2 M sodium cacodylate buffer, postfixed in 1.0% osmium tetroxide, rinsed with the same buffer, and dehydrated in an ethanol series (25%, 50%, 75%, 95%, and 100%) followed by acetone (Benny and Aldrich, 1975). The spores were then embedded in ERL 4206 resin (Spurr, 1969) over a two day period. Isolates tested: L... 906 and GABD 927.

Variation using the same fixation method was tried with spores of GABD 927 which were subjected to previous enzyme digestion. In this case spores were first allowed to germinate in water-agar plates and as soon as the germ tubes emerged they were transferred to vials with a solution of NovoZym™ 234 (Novo BioLabs). The solution was prepared with 0.2g NovoZym, 1 M sorbitol and 50 mM Na citrate. Germinated spores were in this solution for 5 and 18 hr, at room temperature, after which they were fixed as described above.

b) Spores were fixed overnight at 5°C in Karnovsky solution (1965), modified as follow: 2% glutaraldehyde plus 2% paraformaldehyde in 0.2 M sodium cacodylate buffer, washed, postfixed, dehydrated and embedded as described above. Isolates tested: L...925, LCRD 698, and GABD 927.

c) Spores were fixed in 1% osmium tetroxide for 24 hours, washed three times in 0.1 M sodium cacodylate buffer, dehydrated in an alcohol series and directly embedded in LR White, or dehydrated in alcohol followed by acetone and embedded in ERL 4206 resin (Spurr, 1969). Isolates used: AMRW 983, ASCB 984, and GABD 927.

d) Spores were fixed in Karnovsky (1965), modified as described in b) above, for 24 hr at room temperature, washed twice with 0.1 M buffer and twice with distilled water, postfixed with 0.5% aqueous potassium permanganate for 1.5 h at room temperature, washed three times with water, stained *en bloc* with alkaline bismuth for 2 hr at 40°C, followed by an ethanol dehydration series and embedding in LR White resin. The stock solution of alkaline bismuth contained 10% sodium hydroxide, 4% sodium potassium tartrate and 2% bismuth subnitrate; the solution employed was diluted 25 times and kept in an incubator for 15 min at 40°C before being used on the spores. This technique, with slight modifications, has been described by Park et al. (1988) for fixation of other fungal spores. Isolates tested: AMRW 983, LITR 208, and GABD 927.

e) Spores were fixed in 3% potassium permanganate/1 h at room temperature, washed twice in water, dehydrated in an ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%) followed by acetone (Horn, 1989), and embedded in Spurr's resin. Isolate tested: LCRD 698.

f) Spores were quick-frozen (one at a time) in a propane jet-freezer (mod. AE QF1000, H. Aldrich & G. Erdos/ICBR/UF), transferred to glass scintillation vials with 1% osmium tetroxide in absolute acetone and left for 24 hr at -80°C. Vials were successively transferred to -40°C/24 hr, -20°C/overnight, 10°C/2 hr and room temperature/2 hr. Osmium was then removed and the material was washed three times with acetone, followed by infiltration with Spurr's resin (adapted from Aldrich, 1989). This method was tried with isolates L...906 and ASCB 984.

g) Spores were fixed in 7.2% glutaraldehyde in a microwave oven to 40°C (Login and Dvorak, 1985), washed, postfixed, dehydrated and embedded as described in section a) above. Isolate tested: L...906.

h) Spores were fixed in 7.2% glutaraldehyde or in Karnovsky, modified as in b), in a microwave oven to 40°C (Login and Dvorak, 1985). After resting at room temperature/30 min the solution was changed for 20% sucrose plus 30% PEG<sub>400</sub>/30 min. Spores were then transferred to a small glass dish and covered with 30% serum albumin plus 7.2% glutaraldehyde (1:1). Time was allowed for

solidification. The albumin blocks with the spores inside were transferred to a mortar which had been frozen and filled with liquid  $N_2$ . There the blocks were ground until a "powder" was obtained. After warming at room temperature and defrosting, the material was washed with 0.2 M sodium cacodylate buffer. Broken spores found in the serum albumin were selected under a dissecting scope, washed twice in buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer overnight at 5°C. The next day spores were washed three times with buffer and dehydrated with an alcohol series followed by acetone (Benny and Aldrich, 1975). EPON 812 or ERL 4206 resin (Spurr, 1969) were used for embedding, which was processed in two days. Isolates tested: ASCB 984, ALGL 372, GABD 927, GMRG 144, L... 312, L... 925, LCRD 698, L... 906, LITR 208, C...339, and CHTG 400.

In all procedures used, embedded material was kept at 60°C/48 hr, for polymerization. Blocks were sectioned with a diamond knife on a LKB ultramicrotome. The ultrathin sections were collected on one-hole formvar-coated copper grids, post-stained for 40 min in 2% (w/v) aqueous uranyl acetate plus 5 min in Reynolds (1963) lead citrate, and observed on a JEOL-100CX electron microscope at 60 kV. Each method was tested at least twice with the different isolates.

### Results and discussion

Fixation problems are particularly serious with thick-walled, dormant structures like resting sporangia (Olson and Reichle, 1978), oospores (Beakes and Gay, 1977), and zygosporos (Beakes, 1981). This is also the case with spores of arbuscular mycorrhizal fungi, which have thick walls and cannot be fixed and embedded by the usual techniques employed for most fungal structures.

Spore contents of a few arbuscular mycorrhizal species, such as *A. laevis* (Mosse 1970a, 1970b, 1970c) and *Gi. margarita* (Sward 1981a, 1981b, 1981c; Gibson, 1985), have so far been fixed, embedded, and described ultrastructurally. Most of the work that has been done with arbuscular mycorrhizal fungi is related to spore wall structure since fixation of sporoplasm cannot be accomplished easily. When this occurred, with few modifications, the fixative used was acrolein. However, results were not always satisfactory.

In general, all methods and fixatives tested were good for spore walls (Figs. 2.1 - 2.8) but results varied greatly with respect to sporoplasm.

Use of a cell-wall-lysing enzyme on the spores was tried in order to obtain easier penetration of the fixative. NovoZym is a multicomponent preparation used for the production of protoplasts in high yields, and it has been especially useful in the production of protoplasts of

diverse fungi. Although wall degradation had been detected, it did not allow better fixative penetration. Consequently, fixation of sporoplasm was not obtained (Figs. 2.9, 2.10).

Glutaraldehyde has been widely used for fixation of diverse prokaryotes and eukaryotes, since it is very effective for preserving fine structure. A mixture of glutaraldehyde plus formaldehyde has yielded increased depth of good fixation (Hayat, 1981) and has been chosen as the standard control of excellence for immersion fixation (Login and Dvorak, 1985). However, when usual fixations with glutaraldehyde or glutaraldehyde plus paraformaldehyde were tried, spore contents could not be distinguished and membranes were not discernible (Figs. 2.11 - 2.14). Old et al. (1973) used glutaraldehyde plus osmium for fixation of an *Endogone* species, now recognized (Sward, 1981b) as *Scutellospora nigra* (Redheah) Walker & Sanders and were able to fix only the spore wall.

Use of osmium tetroxide did not improve fixation. In fact, sporoplasm was lost and only walls were left at the end of the fixation and embedding processes (Figs. 2.15, 2.16). Osmium tetroxide has been used as a postfixative after using aldehydes. It reacts with unsaturated lipids and certain proteins and gives electron density to the tissues, thus acting as a fixative and as an electron stain. However, it does not react with some sugars and most of the carbohydrates in tissues fixed with osmium are extracted

during rinsing and dehydration (Hayat, 1986). The Golgi complex, multivesicular bodies, and membranous structures are stained well with osmium tetroxide (Hayat, 1986). It is difficult to know why only walls were preserved here.

The same results obtained with glutaraldehyde or the Karnovsky's fixation were also observed when the formaldehyde plus paraformaldehyde combination was followed by potassium permanganate (Fig. 2.17). When 3% potassium permanganate was employed in spores of *Gl. albidum*, spore walls were well preserved. However, in most cases only membranes were distinguished inside the spore (Figs. 2.18, 2.19). Potassium permanganate has been used as post-section stain to increase the electron opacity of cell walls (Hoch, 1977) but in general preservation of cytoplasm is poor. However, Horn (1989) used it successfully for fixation of thick-walled trichospores. Park et al. (1988) obtained better results using alkaline bismuth solution as an *en bloc* stain for spores of *Alternaria alternata* fixed with formaldehyde-glutaraldehyde potassium permanganate.

Although freeze-substitution has been successfully used for fixation of spores of other fungi (Howard and O'Donnell, 1987; Mims et al., 1988), it did not provide good fixation of the spores of arbuscular mycorrhizal fungi tested in this study. Results showed that only spore walls were well preserved while sporoplasm was unclear and apparently damaged (Figs. 2.20, 2.21). The most critical step in this

method is the freezing process (Aldrich, 1989). Variations such as high pressure freezing should perhaps be tried to overcome the main problem of spore fixation, which seems to be the thickness of spore walls and membrane impermeability that act as a barrier to penetration of fixatives and embedding media.

Microwave fixation has been used for histochemical, immunocytochemical, and electron microscope studies (Hopwood et al., 1984, Kok et al., 1988, Wild et al., 1989). In general, excellent preservation of cellular integrity in animal (Login and Dvorak, 1985) and plant tissues (Walsh et al., 1989) can be obtained by this method. The microwave oven generates uniform internal heat across the sample and accelerates diffusion, significantly reducing the processing time. However, in this study microwaving was not adequate to fix the spores. The spore content was unclear and membranes were not delimited (Fig. 2.22). An additional step, the spores being broken in liquid  $N_2$ , was essential to provide fixation. With the combination of microwave plus breaking of spores before postfixation with osmium, good fixation and embedding were obtained with isolates of the genus *Glomus*. The spore wall and sporoplasm were well preserved; nuclei, mitochondria, and other organelles were distinct; and there was little membrane shrinkage (Figs. 2.23, 2.24). Spores of *Gigaspora* and *Scutellospora*, for some unexplained reason, could not be recovered after being

broken in liquid  $N_2$ . It was observed that in spores of *Acaulospora*, although recovered, the fixation of their contents was poor and only walls were well preserved.

One disadvantage of this method is that, after breaking the block with spores, it is very time-consuming to collect the pieces of spores which are mixed with the serum albumin. This seems to be the major problem of the technique, especially when hyaline and very small spores are being fixed. This represents a barrier for fixation of young, newly formed spores. Use of serum albumin proved necessary to act as a pellet, otherwise spores were difficult to break. Another point to be considered is that the good results of the method apply only for *Glomus* species, since spores of neither *Acaulospora*, *Gigaspora*, nor *Scutellospora* were well fixed. Fixation of *Acaulospora* and *Scutellospora* may be more difficult because species in these genera have spores with more than one wall group, while spores of *Glomus* species are characterized by having the walls in one group (Schenck and Pérez, 1990). This explanation, however, cannot be applied to *Gigaspora* species in which the spores also have only one wall group. It could be hypothesized that the problem is with the membranes. Arbuscular mycorrhizal spores must be especially resistant to survive in the rhizosphere environment. If membranes are resistant, impermeable, and very selective, they would act as an effective barrier to avoid damage to spore contents.

Another hypothesis is that penetration of fixatives is blocked by the presence of sporopollenin in the walls of arbuscular mycorrhizal spores. Sporopollenin is a biopolymer considered to be the most resistant organic material (Brooks and Shaw, 1978). It is an oxygenated polymer of carotenoids (Brooks and Shaw, 1978) and has been found in pollen grains of spermatophytes, spores of pteridophytes, in algae, bacteria and some groups of fungi (Gooday, 1981). Among the fungi, presence of sporopollenin has been demonstrated in the zygospore wall of *Mucor mucedo* (Gooday et al., 1973), in sporangiospores, sporangiophores, and zygospores of *Phycomyces blakesleeana* (Furch and Gooday, 1978), in ascospores of *Neurospora crassa*, *N. tetrasperma* (Gooday et al., 1974), and *Ascobolus crenulatus* (Gooday, 1981), and in spores of *Aspergillus niger* and *Penicillium brevi* (Brooks and Shaw, 1977). Sporopollenin was also found in the wall of an arbuscular mycorrhizal fungus, *Gl. versiforme* (Gripiolo and Bonfante-Fasolo, 1984). Apparently sporopollenin is the component of the electron dense line, that separates the primary from the secondary wall of the spore in *Gl. versiforme*. This electron dense line is resistant to alkali extraction and acetolysis (Gripiolo and Bonfante-Fasolo, 1984). While performing biochemical studies with different species of arbuscular mycorrhizal fungi, Weijman and Meuzelaar (1979) noticed that some spores and sporocarps of *Glomus* and

*Endogone* were "...unusually resistant to hydrolysis in 1N HCl." They suggested that such characteristics may be due to melanin deposition in the cell walls. However, this could also be the result of the presence of sporopollenin. Walls containing sporopollenin probably have additional resistance to physical, chemical, and microbiological degradation. In that way it is possible that sporopollenin occurs as a common constituent of arbuscular mycorrhizal spores, protecting them against degradation in adverse conditions. The fact that good fixation of *Glomus* spores had been obtained only after breaking them in liquid  $N_2$ , and the comments of Mosse (1970b) about piercing the spores of *A. laevis* to get better fixation, seem to indicate that this hypothesis could be true. However, not all genera of arbuscular mycorrhizal fungi have spores with an electron-dense line between the primary and secondary walls. More research in this area is needed in order to identify the elements that constitute the spore walls of the different genera of arbuscular mycorrhizal fungi.

The results obtained in this research showed that there are few techniques which provide satisfactory fixation and embedding of arbuscular mycorrhizal spores. It will be necessary to find a different fixation procedure for each genus, or even species studied, since the response to fixation and embedding processes has been different among the taxa.

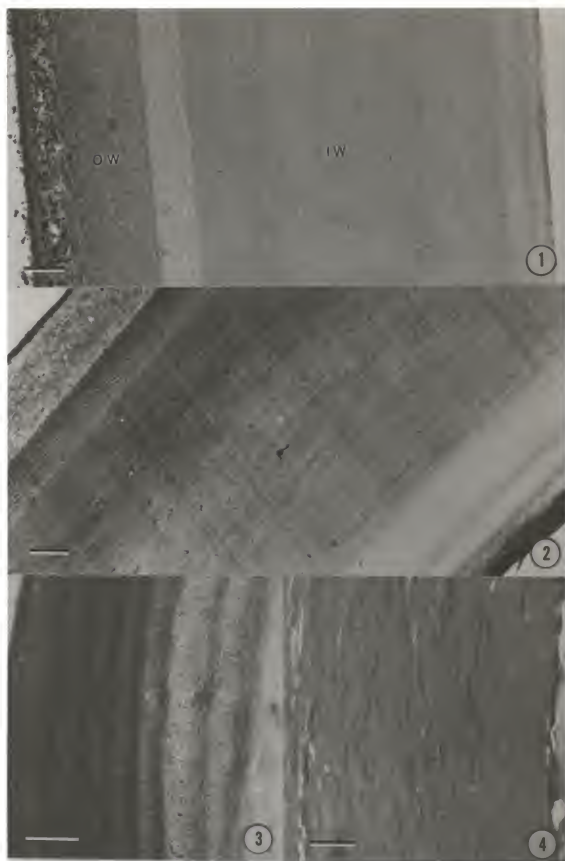
Fig. 2.1. - 2.4. Transmission electron micrographs of spore walls of some arbuscular mycorrhizal fungi fixed by different techniques.  
Abbreviations: IN= inner wall; OW= outer wall. (Bars = 1  $\mu$ m).

Figure 2.1 - *Gigaspora albida* (GABD 927) - fixation: Karnovsky + potassium permanganate.

Figure 2.2 - *Gigaspora albida* (GABD 927) - fixation: glutaraldehyde + osmium after 5 hr of enzyme digestion.

Figure 2.3 - *Acaulospora morrowiae* (AMRW 983) - fixation: osmium/24 hr.

Figure 2.4 - *Glomus claroides* (LCRD 698) - fixation: 3% potassium permanganate.



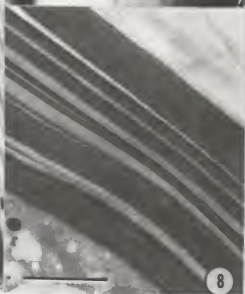
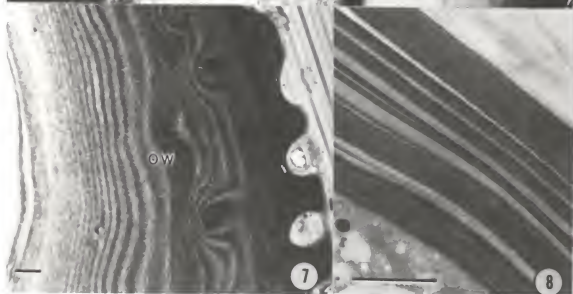
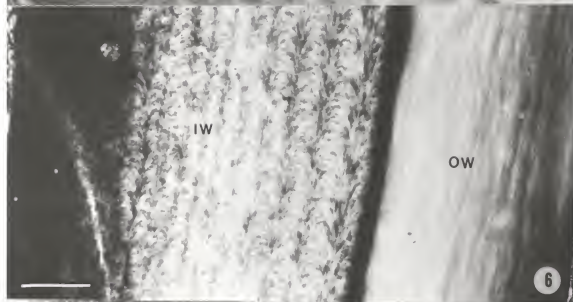
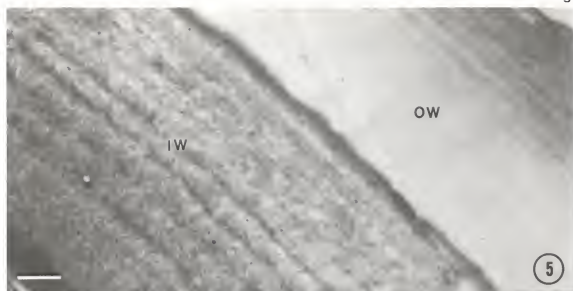
Figs. 2.5. - 2.8. Transmission electron micrographs of spore walls of some arbuscular mycorrhizal fungi fixed by different techniques.  
Abbreviations: IN= inner wall; OW= outer wall. (Bars = 1  $\mu$ m).

Figure 2.5 - *Glomus* sp. (L... 209) - fixation: Karnovsky + osmium.

Figure 2.6 - *Glomus* sp. (L... 906) - fixation: freeze substitution + osmium.

Figure 2.7 - *Acaulospora scrobiculata* (ASCB 984) - fixation: glutaraldehyde in a microwave oven to 40°C, broken in liquid N<sub>2</sub> and postfixed in osmium.

Figure 2.8 - *Glomus intraradices* (LITR 208) - fixation: glutaraldehyde in a microwave oven to 40°C, broken in liquid N<sub>2</sub> and postfixed in osmium.



Figs. 2.9 - 2.14. Transmission electron micrographs of spores of some arbuscular mycorrhizal fungi fixed by different techniques. Abbreviations: L= lipid; N= nucleus. (Bars = 0.5  $\mu$ m).

Figure 2.9 - *Gigaspora albida* (GABD 927) - fixation: glutaraldehyde + osmium after 5 hr of enzyme digestion.

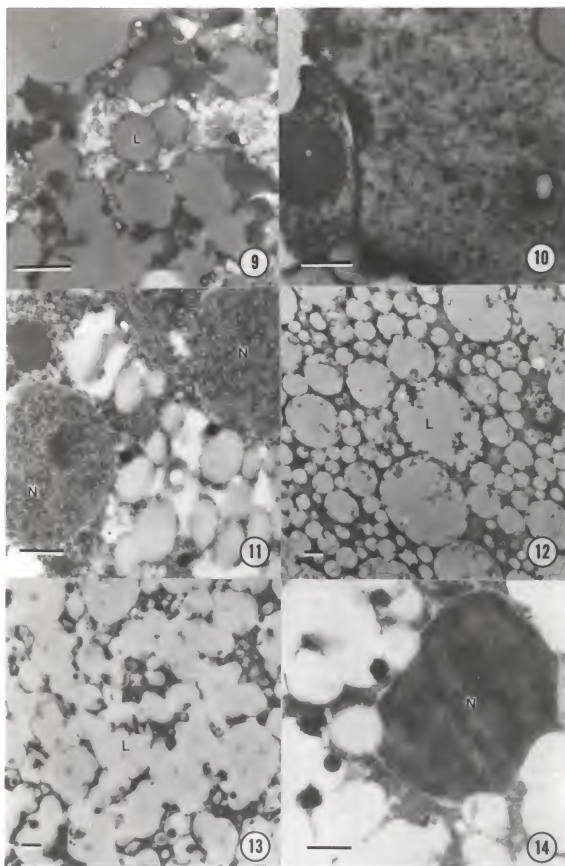
Figure 2.10 - *Gigaspora albida* (GABD 927) - fixation: glutaraldehyde + osmium after 17 hr of enzyme digestion.

Figure 2.11 - *Glomus* sp. (L... 906) - fixation: glutaraldehyde + osmium.

Figure 2.12 - *Glomus* sp. (L... 906) - fixation: glutaraldehyde + osmium.

Figure 2.13 - *Gigaspora albida* (GABD 927) - fixation: Karnovsky + osmium.

Figure 2.14 - *Gigaspora albida* (GABD 927) - fixation: Karnovsky + osmium.



Figs.2.15. - 2.19. Transmission electron micrographs of spores of some arbuscular mycorrhizal fungi fixed by different techniques. Abbreviation: W=wall.

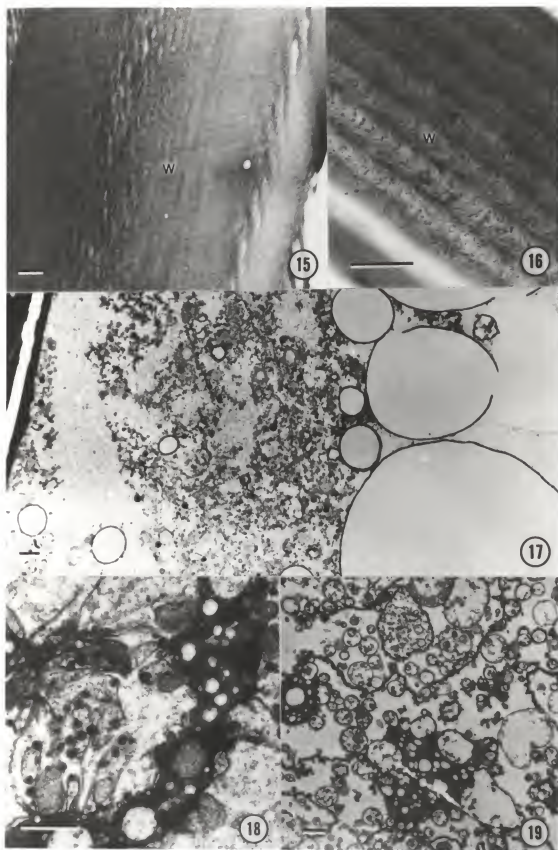
Figure 2.15 - *Gigaspora albida* (GABD 927) - fixation: osmium/24 hr. (Bar= 0.5  $\mu$ m).

Figure 2.16 - *Acaulospora morrowiae*. (AMRW 983) - fixation: osmium/24 hr. (Bar= 0.5  $\mu$ m).

Figure 2.17 - *Gigaspora albida* (GABD 927) - fixation: Karnovsky + potassium permanganate. (Bar= 1  $\mu$ m).

Figure 2.18 - *Glomus claroides* (LCRD 698) - fixation: 3% potassium permanganate. (Bar= 0.5  $\mu$ m).

Figure 2.19 - *Glomus claroides* (LCRD 698) - fixation: 3% potassium permanganate. (Bar= 0.5  $\mu$ m).



Figs. 2.20. - 2.24. Transmission electron micrographs of sporoplasm of *Glomus* sp. (L... 906) fixed by different techniques. Abbreviations: B= bacteria-like organelle (BLO); L= lipid; M= mitochondria; N= nucleus. (Bars= 0.5  $\mu$ m).

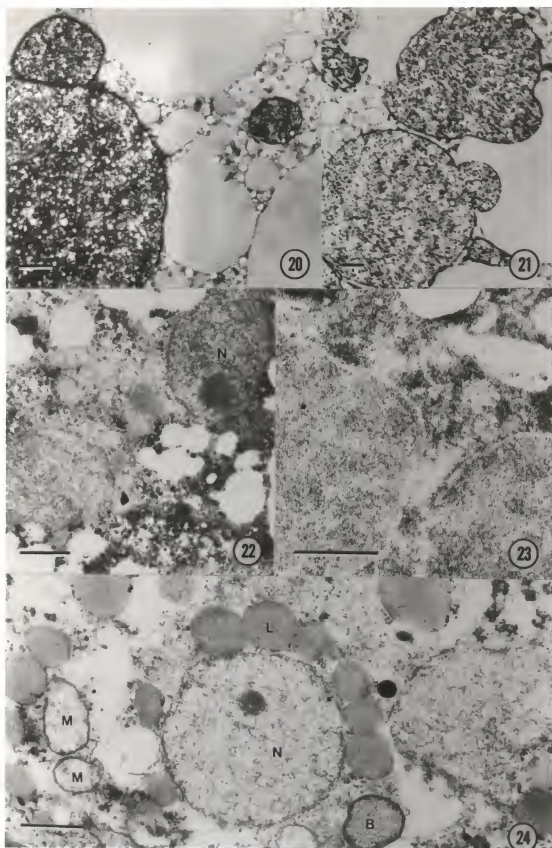
Figure 2.20. - *Glomus* sp. (L...906) fixed by freeze substitution + osmium.

Figure 2.21 - *Glomus* sp. (L...906) fixed by freeze substitution + osmium.

Figure 2.22 - *Glomus* sp. (L...906) fixed with glutaraldehyde in a microwave oven to 40°C and postfixed with osmium.

Figure 2.23 - *Glomus* sp. (L...925) fixed with glutaraldehyde in a microwave oven to 40°C and postfixed with osmium.

Figure 2.24 - *Glomus* sp. (L...906) fixed with glutaraldehyde in a microwave oven to 40°C, broken in liquid N<sub>2</sub> and postfixed with osmium.



CHAPTER III  
ULTRASTRUCTURAL STUDIES OF SPORE WALLS  
AND ASSOCIATED BACTERIA

Introduction

Wall Structure

Classification of arbuscular mycorrhizal fungi is based mainly on morphological characters of the spore. Apparently the most consistent of those features are related to developmental functions, such as type, number, and position of spore walls, events in spore development and germination processes (Morton, 1988). These characters remain structurally constant even under diverse environmental conditions (Morton, 1985, 1990a). It has been shown that instead of being an inert cover for cells, the cell walls are active, adaptable, and sensitive to the surrounding environment (Bonfante-Fasolo, 1988). Weijman and Meuzelaar (1979) considered the cell wall as "... a highly complex, polyfunctional and metabolically active" component of the cell. As defined by Bartnicki-Garcia (1968), "The wall, more than any cellular part, defines a fungus and distinguishes it from other living creatures." In fact, wall structure has also been considered an important evolutionary character because of its stability and importance in spore development and function (Bartnicki-

Garcia, 1968). In a study of evolutionary relationships among arbuscular mycorrhizal fungi, from all characters considered, Morton (1990b) placed major emphasis on components of wall structures.

Gerdemann and Trappe (1974) were the first to recognize the importance of spore wall structure to separate and identify species of arbuscular mycorrhizal fungi. Subsequently, a new concept of spore wall structure which would help the standardization of species description in this group of fungi was created by Walker (1983). He described four wall types: evanescent, unit, laminated, and membranous walls. Later, other wall types were described: the coriaceous wall (Walker, 1986), the expanding wall (Berch and Koske, 1986), the amorphous wall (Morton, 1986a), and the germinal wall (Spain et al., 1989). Morton (1986a) developed a murograph for the beaded wall which had been introduced by Schenck et al. (1984). Schenck and Pérez (1987) revised the murograph of the expanding wall. Detailed explanations of these wall types with examples of genera and species in which they occur are given by Morton (1988).

Spore walls proved to be useful taxonomic characters and consequently have been widely used as diagnostic features to identify species within genera of arbuscular mycorrhizal fungi (Bonfante-Fasolo and Schubert, 1987; Hall, 1984; Morton, 1988; Schenck and Pérez, 1990; Spain, 1990;

Trappe and Schenck, 1982; Walker, 1983). Bonfante-Fasolo and Schubert (1987) showed that the spore wall is a reliable taxonomic criterion for identification of arbuscular mycorrhizal species. However, the wall structure in these fungi has been the object of much discussion since the number and structure of wall and wall layers is often difficult to observe with the light microscope (LM), leading to incomplete descriptions and/or misinterpretations. Moreover, use of mountants and preservatives usually change wall characters (Berch and Koske, 1986; Koske and Walker, 1986; Morton, 1986b). Descriptions based on such data do not represent the natural morphology of the species as observed when the spores are suspended in water (Spain, 1990). Interpretation of wall structure at the LM level has been difficult due to lighting, mounting medium, and personal interpretation (Mosse, 1986). In addition, one should consider that the apparent degree of cohesion between spore walls and layers, and the way they can be physically and optically separated will depend on spore age, rate of development and growth conditions (Mosse, 1986; Morton, 1988). Light microscope studies remain, however, as the most accessible and practical way to identify arbuscular mycorrhizal species. On the other hand, TEM can provide more accurate information about wall structure and, as a supplement to LM observations it may confirm and clarify taxonomic concepts. Moreover, in association with bio-

chemical studies, it may be useful in demonstrating phylogenetic relationships (Mosse, 1986). Gibson (1985) was able to demonstrate that outer walls of *Gigaspora* spp., *Scutellospora* spp. and *Gl. etunicatum* are similar to the zygosporangium of *Endogone pisiformis* Link: Fr. in staining properties which he suggested may indicate some degree of evolutionary relationship between the orders Glomales and Endogonales.

The impact of wall studies on taxonomic problems has been questioned by Mosse (1986), who emphasizes the need for more information about spore wall structure at the TEM level. She discussed the ultrastructure of spore walls in some arbuscular mycorrhizal species, including *Gl. mosseae* (Nicol. & Gerd.) Gerd. & Trappe, *Gl. caledonium* (Nicol. & Gerd.) Trappe & Gerd., *Scutellospora* sp. (as *Gigaspora* sp.), and *A. laevis*. Mosse (1970a, 1970b, 1970c) published a detailed ultrastructural study about development of spores in *A. laevis*. She described the spore wall in this species as formed by an outer wall which is fibrillar and multilayered, with each layer showing a characteristic arc shaped pattern of fibrils, and a membranous, structureless inner wall. Moreover, there is a thin, electron dense line between the inner and the outer wall which she named the "separation layer" (Mosse, 1986). This layer has a series of radially oriented lines. Sward (1981a) mentioned the same structure in the spore wall of *Gi. margarita*. Mosse

(1986) described two more inner walls in *A. laevis* with a granular appearance. The *Scutellospora* species she described showed, with the LM, a single, sometimes laminated wall. With TEM, four walls were distinguished: the first, or outer, is fibrillar, the second is multilayered; the third is structurally similar to the first, and the fourth is thin and electron transparent. This species was not identified but resembles *Scutellospora gilmorei* (Trappe & Gerd.) Walker & Sanders (Mosse, 1986).

Old et al. (1973) described LM, SEM, and TEM studies of a species from Nigeria, later identified as *Scutellospora nigra*, which at that time they thought was a new species of *Endogone*. The spore wall showed a complex structure with a dark outer wall formed by two layers: an inner layer of open spiral meshwork, and an outer layer, with a regular pattern of pores. The inner wall was apparently formed by four distinct layers. The authors compared the spore ultrastructure of this species with that of *A. laevis* (Mosse, 1970c) and concluded that three features were present in both species: a) a layer with radial periodicity, b) the development of a sectorial wall layer, and c) the formation of pregermination compartments in the periphery of the spores. This type of germination, characterized by formation of peripheral compartments, was described in detail by Mosse (1970a), and it has been found in other

arbuscular mycorrhizal species (Gerdemann and Trappe, 1974; Hall, 1977; Mosse and Bowen, 1968; Sward et al., 1978).

Description of spore wall structure of *Gl. versiforme* was given by Bonfante-Fasolo and Vian (1984), who first demonstrated a regular organization of microfibrills in ordered layers in spore walls of an arbuscular mycorrhizal fungus. This organization had been observed in other mycorrhizal species but apparently it was not understood at that time. Mosse (1970a) mentioned only a "curved fibrillar substructure, alternating with narrow bands of tangentially oriented fibrils" in the spore wall of *A. laevis*. Later (Mosse, 1986) she described it as a multilayered, fibrillar layer with the appearance of arced fibrils. Bonfante-Fasolo and Vian (1984) showed that the spore wall in *Gl. versiforme* is complex, multi-layered and contains a regular pattern of wall subunits. There are in fact two walls: the outer wall which consists of a single layer of parallel microfibrils, and an inner wall with two layers of different organization. Thus, three different wall textures were present: a) an ordered texture, composed by parallel microfibrils, in the outer wall; b) an arced texture, made up by overlapping layers of straight microfibrils, in the inner wall; c) a dispersed texture, without clear organization, in the innermost part of the inner wall. In this paper, Bonfante-Fasolo and Vian (1984) pointed out the occurrence of the arced organization, a special feature which had only been

reported in algal walls (Pearlmutter and Lembi, 1978), in primary and secondary walls of higher plants (Vian, 1978), and in the cuticle of Crustacea (Livolant et al., 1978) and Insecta (Filshie, 1982). Thus, although widespread in nature, this typically ordered microfibrillar organization is not common among the fungi. The exceptions are some species of Glomales and one species of Ascomycetes, *Limacinula theae* Syd. & Butl., where similar organization was suggested to occur in its bitunicate asci (Reynolds, 1971). In Glomales, many species were shown to have this special type of arrangement: *Gl. constrictum* Trappe, *Gl. etunicatum* (Gibson, 1985), *Gl. versiforme* (Bonfante-Fasolo and Grippio, 1984; Bonfante-Fasolo and Vian, 1984), *Gl. macrocarpum* Tul. & Tul. and *Gl. caledonium* (Nicol. & Gerd.) Trappe & Gerd. (Bonfante-Fasolo and Schubert, 1987), *Scutellospora* sp. (Mosse, 1986), *Scutellospora pellucida* (Nicol. & Schenck) Walker & Sanders (Gibson, 1985), and *A. laevis* (Mosse, 1970c, 1986).

The helicoidal texture, showing curved fibrils, is an artifact (Neville and Levy, 1985; Roland and Vian, 1979), according the model first proposed by Bouligand (1965) and later widely accepted (see revision in Filshie, 1982). The explanation for the so-called "helicoidal model" of Bouligand which is evident as bow-shaped lines in the wall, is that there are stacked layers with straight fibrils regularly organized in parallel. Each layer is rotated at a

small angle with respect to the previous one, and the arced appearance is produced by overlapping planes of fibrils (Bonfante-Fasolo and Schubert, 1987; Bonfante-Fasolo and Vian, 1984; Filshie, 1982; Roland and Vian, 1979). The arcs seen in sections are the result of a progressive rotation of the microfibrils with time (Vian and Roland, 1987). The illusory nature of the arced texture can be demonstrated by tilting, which produces changes in appearance, such as cancellation or reversal of the arcs (Roland and Vian, 1979). The development of thick helicoidal walls as occurs in spores of some arbuscular mycorrhizal fungi makes the cell mechanically very hard by nature and, consequently, more difficult to study (Vian and Roland, 1987).

Comparing light and electron microscopy studies on different *Glomus* species (*Gl. versiforme*, *Gl. macrocarpum*, *Gl. caledonium*, *Gl. clarum*, *Gl. occultum* Walker, *Gl. constrictum*, *Gl. mosseae*, and *Gl. fasciculatum*), Bonfante-Fasolo et al. (1985) and Bonfante-Fasolo and Schubert (1987) concluded that there is an agreement between LM and TEM observations. More than that, they affirm that the wall constitutes a "...trustable taxonomic criterion for identifying VAM spores." The authors based their conclusion on the fact that all species examined had a different wall architecture, which indicates a diverse pattern of fibril deposition during spore morphogenesis. However, they also mentioned that some features, such as a fibrillar texture,

were shared by all spores examined. Spore walls of *Gl. macrocarpum* are formed by two layers: an outer, thin, evanescent layer, and an inner, thick, laminated layer, showing at the TEM level a typical helicoidal structure. In *Gl. clarum*, there are two closely attached wall layers, with different texture, separated by an electron dense line. The outer layer is formed by disorganized material while the inner layer is formed by ordered fibrils embedded in an amorphous matrix. The spores of *Gl. mosseae* have two different walls which are easily separated. Both are formed by ordered fibrils embedded in an electron-dense material; but are different in that the dense material is reduced in the outer layer. In *Gl. fasciculatum* there are also two wall layers which are characterized by a homogeneous texture. In contrast, spore walls of *Gl. caledonium* are formed by two wall layers with different organization: the outer layer has ordered microfibrils; the inner shows a layered organization with a parallel deposition in which the arced appearance is evident. These descriptions of *Gl. mosseae* and *Gl. caledonium* spore walls ultrastructure are in agreement with those presented by Mosse (1986). *Glomus constrictum* has walls with two layers, an inner layer of fibrillar texture, and an outer with two parts, the external sloughing into the soil, and the internal of unordered fibrillar organization. In his description of *Gl. constrictum* spore walls, Gibson (1985) showed that, in

contrast, the inner wall, which he considered the outer wall, is made up of microfibrils in an apparent arcuate orientation. He mentions that an outer wall layer was not evident, except for a very thin outermost zone. In *Gl. occultum* the spore wall is formed by two easily separable layers with fibrils embedded in an electron-dense matrix (Bonfante-Fasolo and Schubert, 1987). The spore wall of *Gl. etunicatum* was studied by Gibson (1985), who described it as formed by two walls, one ephemeral, which appears to be divided into two layers, and a persistent, inner wall, where an arcuate pattern of microfibrils is suggested but not evident on his TEM micrographs.

Spore walls of *Gi. margarita* have been described at the ultrastructural level by Sward (1981a) and Gibson (1985). There are some disagreements between these two descriptions. Sward (1981a) describes the wall as formed by four layers: an outer layer that easily separates from the other layers, a narrow layer with striations of regular periodicity, a thick wall layer composed of many laminations, and a fourth and innermost layer of varied thickness. Gibson (1985) mentions a thin outer wall layer, with more or less uniform texture of parallel microfibrils, an inner, much thicker inner wall layer, which is characterized by formation of bands with apparent arcuate pattern of microfibrils, and an innermost, thin, electron opaque wall. He also showed that differences in staining treatment of the thin sections may

enhance or decrease staining properties of certain spore wall layers.

Spores of *Gi. gigantea* (Nicol. & Gerd.) Gerd. & Trappe have two walls: an outer wall, formed by a thin outermost layer with two zones, and an inner thicker layer of parallel microfibrils, and an inner thick wall, showing fibrils with a perpendicular orientation (Gibson, 1985).

Ultrastructural studies of spore walls were also performed by Gibson (1985) with three species of *Scutellospora*: *S. pellucida*, *S. heterogama*, and *S. gregaria*. The spores of *S. heterogama* were described as having three walls: an outer wall with three distinct sublayers, a membranous inner wall, composed by two sublayers, which are separated by a double membrane-like partition, and a thin, innermost layer which frequently is sublayered. In *S. pellucida*, the spore has these distinct walls: an outer, with many thin lamellae, which at high magnifications appear as bands of microfibrils in an arcuate pattern, a membranous inner wall, formed by two distinct layers of similar thickness, and an innermost layer. The outer wall and the membranous wall are separated by a double membrane-like partition, as occurs in spore walls of *S. heterogama*. Spores of *S. gregaria* have an outer, ornamented wall, divided in two layers, and an inner wall formed by a thick outer layer with fine, radial striations, and a thinner inner layer. It is important to notice that these species

were previously classified as *Gigaspora* and were treated as such by Gibson (1985). Subsequently, they were transferred to the new genus *Scutellospora* Walker & Sanders (1986) who considered the formation of germination shields in species with inner flexible walls, in opposition to species that do not have inner flexible walls and germinate directly through the spore wall as the main character for separating *Gigaspora* into two genera. The ultrastructural studies of Gibson (1985) confirmed these differences. He mentioned specifically that the structure of the wall in *Gigaspora* species falls into two broad categories: those with and those without an inner membranous wall, and that this was correlated with the two different types of germination observed in the group. Gibson (1985) also attempted to study *Sclerocystis coremioides* but concluded that the integration of spore walls with the surroundings hypha in the sporocarp made it difficult to interpret the wall organization. Studies of this type show the importance of the ultrastructural observation as an important aid in taxonomy.

#### Cell Wall Components

Fungal cell walls are composed of polysaccharides (80 to 90%), protein and lipid. Pigments, polyphosphate, and inorganic ions may also be present. Physically the walls are formed by interwoven microfibrils embedded in or

cemented together by an amorphous matrix. Proteins and diverse polysaccharides (glucans, mannans, etc.) are known to constitute this cementing matrix (Bartnicki-Garcia, 1968).

The most important structural component of most fungal cell walls is chitin, a homopolymer of N-acetylglucosamine (Lopez-Romero and Ruiz-Herrera, 1986). In Zygomycetes, chitin can be partly deacetylated to chitosan and linked to peptides (Sietsma et al., 1986). Another component which may occur in the matrix of the zygomycete wall is polyglucuronic acid (Gooday, 1989). In *Mucor rouxianus* (Calmette) Wehmer (as *M. rouxii*), glucuronic acid constitutes 25% of the sporangiophore wall (Bartnicki-Garcia, 1968). Presence of abundant residues of N-acetylglucosamine has been reported in cell walls of *Gl. clarum* (Jabaji-Hare et al., 1990) and ultrastructurally localized in cell walls of *Gi. margarita* (Grandmaison et al., 1988). Spore walls of *Gl. versiforme* have chitin as the major component, with glucans, uronic acids, alkali soluble proteins and alkali soluble sugars also present. Another spore wall constituent found in this species was sporopollenin, an extremely resistant polymer of carotenoids (Bonfante-Fasolo and Grippiolo, 1984). In fungi, sporopollenin has been found in reproductive structures of some Zygomycotina and in spores of a few Ascomycotina (Brooks and Shaw, 1977; Furch and Gooday, 1978; Gooday,

1981; Gooday et al., 1973, 1974). Investigations on other *Glomus* and *Gigaspora* species have also shown that walls of arbuscular mycorrhizal fungi are composed essentially of chitin (Weijman and Meuzelaar, 1979). Sialic acid and fucose residues were detected in cell walls of all structures formed by *Gl. clarum* (Jabaji-Hare et al. 1990). Fucose is known to be present in other fungi, particularly in Mucorales (Zygomycetes) and Basidiomycetes (Bartnicki-Garcia, 1968). Sialic acid may occur in the cell wall as a terminal residue of a polysaccharide or glycoprotein (Jabaji-Hare et al. 1990). Presence of chitin, sporopollenin, fucose and uronic acids in the cell wall supports the classification of Glomales among the Zygomycetes and may perhaps indicate close relationship with the Mucorales.

#### Cell Wall Associated Bacteria

The rhizosphere represents the most biologically active part of the soil (Vancura, 1986). In the root environment microorganisms are influencing each other in many different ways and a synergistic interaction between endomycorrhizal fungi and rhizosphere microorganisms is suppose to exist (Azcón, 1989). Direct evidence of microbe-microbe mutualistic relationships involving arbuscular mycorrhizal fungi have been reported by many authors (Ames et al., 1984; Azcón-Aguilar and Barea, 1985; Azcon-Aguilar et al., 1986;

Barea et al. 1983; Krishna et al., 1982; Manjunath et al., 1981; Mayo et al., 1986). Mycorrhizal fungi are known to influence other microorganisms in the rhizosphere, either through a direct effect on root physiology with consequent changes in root exudation or through direct effects of their exudates (Paulitz and Linderman, 1989). This concept of "mycorrhizosphere effect" has been discussed by Lindermann (1988, 1990) and Paulitz and Lindermann (1990). However, the mechanisms that regulate the influence of arbuscular mycorrhizal fungi on microbial populations are still unknown (Ames et al., 1984).

Azcón (1989) observed that microorganisms living in the rhizosphere exert a stimulatory effect on the area around the root, a natural habitat for arbuscular mycorrhizal fungi. From his studies, he concluded that there is a selective effect of arbuscular mycorrhiza and bacteria on their mutual development. In fact, the root surface usually maintains bacterial colonies which can extend beyond the mycorrhizal zone (Fasolo-Bonfante and Scannerini, 1975). Increase in bacterial population has been observed soon after plants become mycorrhizal (Ames et al., 1984; Bagyaraj and Menge, 1978), but in some cases a significant increase does not persist (Azcón et al. 1976; Barea et al. 1975). Populations of bacteria and actinomycetes were found to be larger in the rhizosphere of mycorrhizal versus non mycorrhizal tomato plants (Bagyaraj and Menge 1978). On the

other hand, Paulitz and Linderman (1989) demonstrated for the first time that some species of arbuscular mycorrhizal fungi affect the population of fluorescent pseudomonads introduced as seed treatment and which are used as biocontrol agent of various plant diseases. There are also indications that the composition of species of rhizosphere microorganisms may be altered by mycorrhizal fungi (Ames et al., 1984). Azcón (1989) reports stimulation of arbuscular mycorrhizal spore germination and root colonization by saprophytic bacteria and, at the same time, stimulation of bacterial growth in presence of arbuscular mycorrhizal fungi. Stimulation of spore germination of arbuscular fungi by soil microorganisms has also been reported (Daniels and Trappe, 1980). In a study on "independent" growth of arbuscular endophytes, Mosse (1988) used root fragments as starting material and found that none of the cultures were free from bacterial contamination. Among the conclusions, she could not determine if the observed independent growth was "...due to the presence of transformed roots, to the bacterial contamination or to a combination of these factors." When studying inhibition of spore germination of some endomycorrhizal fungi, Tommerup (1985) showed that bacteria from the spore surface stimulated germination when that was blocked on sterile extracts of soils. Apparently the bacteria act by inactivating inhibitory compounds in soil or by a direct effect on the spore, promoting its

potentiality to overcome the inhibition. Sylvia and Schenck (1983) found that soil pasteurization did not affect spore germination of certain arbuscular mycorrhizal species probably because the beneficial microorganisms, which are likely to be spore-forming bacteria or Actinomycetes, can survive this treatment. Spore germination and hyphal growth of *Gl. versiforme* were shown to be significantly greater when spore-associated bacteria were present. Some of these bacteria were of different genera, including *Corynebacterium* and *Pseudomonas* (Mayo et al., 1986). Tylka et al. (1991) studied the effect of three *Streptomyces* species on germination of arbuscular mycorrhizal spores. They found that germination of *Gi. margarita* and *Gl. mosseae* was stimulated by volatile compounds from *Streptomyces orientalis*. *Glomus mosseae* spore germination was also stimulated by the other species of *Streptomyces* tested, *St. avermitilis* and *St. griseus*. The stimulatory effect of *Streptomyces* species on germination of *G. mosseae* had been reported before (Mugnier and Mosse, 1987). Conversely, germination of spores of *Scutellospora heterogama* was stimulated by *St. orientalis* only when physically separated from the medium containing the organism, and suppressed in the presence of *St. avermitilis* or *St. orientalis*. It was hypothesized that germination was inhibited by the increase in pH produced by the colonies of *Streptomyces* on the culture medium (Tylka et al. 1991).

It has been demonstrated that chitin-decomposing actinomycetes present on spores of arbuscular mycorrhizal fungi can enhance mycorrhizal development and growth of onion (Ames, 1989). Apparently there is a prevalence and high diversity of chitin-decomposing actinomycetes associated with spores of arbuscular mycorrhizal fungi in field soils (Ames et al., 1989). In the same study they mentioned that of the 190 spores of *Gl. macrocarpum* examined, 100 were infected by one or more chitin-decomposing microorganism (82% were colonized by actinomycetes, 17% by bacteria, and 1% by fungi). Presence of such actinomycetes should not be a surprise since spore walls of arbuscular mycorrhizal fungi have a large amount of chitin (Bonfante-Fasolo and Grippiolo, 1984; Bonfante-Fasolo et al., 1986) and therefore, constitute a rich substrate for chitin-decomposing actinomycetes (Williams et al., 1984).

Colonization of spore walls of arbuscular mycorrhizal fungi by bacteria has been shown at the ultrastructural level in many species such as *Gl. clarum*, *Gl. constrictum*, *Gl. macrocarpum* (Bonfante-Fasolo and Schubert, 1987), *Gl. intraradices*, *Gl. etunicatum* (Gibson, 1985), and *Gl. caledonium* (MacDonald and Chandler, 1981). Actinomycete-like organisms were shown by SEM on the spore wall of *Gi. candida* Bhattacharjee, Mukerji, Tewari & Skoropad (Bhattacharjee et al., 1982). In all of these studies in which bacteria have been noticed, there is no evidence of

the impact they may be having on arbuscular mycorrhizal spores.

Although a broader knowledge about walls of some arbuscular mycorrhizal fungi is now available, there are still many points which need to be clarified. For example, how are the walls in spores of arbuscular mycorrhizal species formed, how is the development of these walls related to development of spore walls in other Zygomycetes, and which wall features are more reliable in showing relationships among the families of Glomales and between this order and other groups of Zygomycetes? The answers to these questions would be of real value for better understanding the position of Glomales among the Zygomycetes.

The purpose of this work is to provide more information about wall development and wall structure of arbuscular mycorrhizal fungi in order to apply this knowledge to the study of the phylogenetic relationship of the Glomales with the Endogonales and other groups of Zygomycetes.

### Materials and Methods

#### Source of Materials

Spores and attached structures of the species listed below were selected from pot cultures or from aeroponic culture as described in Chapter II.

Isolates studied:

*Acaulospora morrowiae* (INVAM - AMRW 983)

*A. scrobiculata* (INVAM - ASCB 984)

*Glomus* sp. (INVAM - L... 312)

*Gl. claroides* (INVAM - LCRD 698)

*Glomus* sp. (INVAM - L... 925)

*Glomus* sp. (INVAM - L... 906)

*Gl. intraradices* (INVAM - LITR 208)

*Gigaspora albida* (INVAM - GABD 927)

Light Microscopy (LM)

Vegetative and reproductive structures of different species of arbuscular mycorrhizal fungi were selected from Petri dishes where they had been maintained in water, at 4°C, mounted on a microscope slide either in water, Melzer's or PVLG (polyvinyl alcohol-lactic acid-glycerol - Koske and Tessier, 1983), and observed directly with a Nikon compound microscope.

Scanning Electron Microscopy (SEM)

Spores and respective attachments were prepared for SEM in two different ways: a) material from isolates GABD 927, LCRD 698, and L... 925, was fixed in Karnovsky (1965), modified as described in Chapter IIb, post fixed with 0.1% osmium tetroxide, washed with 0.1 M sodium cacodylate buffer, dehydrated in an ethanol series, transferred to

small glass vials with hexamethyldisilazane (HMDS - Polyscience) for 5 min and left overnight in a hood. After drying, spores were placed on stubs with double-sticky cellophane tape, coated with gold in a Hummer Jr. sputter coater and observed in a Hitachi S-450 scanning electron microscope. Spores of *Gl. intraradices* (LITR 208) were prepared in the same way but together with the pieces of roots were they had been formed.

b) Spores from isolates ASCB 984, and L... 906 were selected, transferred to small glass vials, covered with HMDS for 5 min and left for drying overnight in a hood, after which they were gold coated as described above and observed in a Hitachi S-456 scanning electron microscope.

#### Transmission Electron Microscopy (TEM)

Preparations for TEM were the same as described in Chapter II. Specifications of the fixation process used for each isolate are given below:

*Acaulospora morrowiae* (AMRW 983) - spores fixed in 1% osmium tetroxide/24 hr as described in Chapter II (c);

*Acaulospora scrobiculata* (ASCB 984) - spores prepared by freeze substitution, as described in Chapter II (f) or fixed with glutaraldehyde in a microwave oven, broken in liquid N<sub>2</sub>, and post-fixed in 1% osmium tetroxide, as described in Chapter II (h).

*Glomus* sp. (L... 312) - spores fixed with Karnovsky (1965), modified as described in Chapter II (b), broken in liquid N<sub>2</sub>, and post-fixed with 1% osmium tetroxide as described in Chapter II (h).

*Glomus claroides* (LCRD 698) - spores fixed in 3% potassium permanganate as described in Chapter II (e);

*Glomus* sp. (L... 925) - spores fixed in Karnovsky (1965), modified as described in Chapter II (b), or fixed in glutaraldehyde in a microwave oven, broken in liquid N<sub>2</sub>, and post-fixed with 1% osmium tetroxide as described in Chapter II (h);

*Glomus* sp. (L... 906) - spores prepared by freeze-substitution, as described in Chapter II (f) or fixed with glutaraldehyde in a microwave oven, broken in liquid N<sub>2</sub>, and post-fixed with 1% osmium tetroxide as described in Chapter II (h);

*Glomus intraradices* (LITR 208) - spores were fixed with glutaraldehyde in a microwave oven, broken in liquid N<sub>2</sub>, and postfixed with 1% osmium tetroxide as described in Chapter II (h);

*Gigaspora albida* (GABD 927) - spores were fixed with glutaraldehyde after enzyme digestion, as described in Chapter II (a), or fixed in Karnovsky solution (1965) and postfixed with 1% osmium tetroxide, as described in Chapter II (b), or fixed with Karnovsky (1965) followed by 0.5% potassium permanganate, as described in Chapter II (d).

Samples of all isolates were embedded, thin sectioned, collected in one hole copper grids, post-stained and observed in a JEOL-100CX electron microscope, as described in Chapter II.

### Bacterial Isolation

Spores of species which had been found colonized by bacteria were placed on Petri dishes with 1.5% water agar. Inoculum from the bacterial colonies which developed around the spores were transferred to other plates with 1.5% water agar and 0.25% chitin (as powder of crustacea shells). The colonies of supposedly chitin degrading bacteria that developed there were transferred to nutrient agar and prepared for identification through the fatty acid profile test of similarity (Fatty acid Library/Plant Pathology Department/University of Florida).

### Results and Discussion

The wall terminology used in this work follows the recommendations of Walker (1983), Morton (1988) and Schenck and Pérez (1990). Thus, for clarification walls will be classified in groups (= adherent aggregation of walls that do not readily separate when the spore is broken - Walker, 1983), and each group may contain one to several walls of different types. To better explain details at the

ultrastructural level, in some instances the term "layer" has been used here to describe stratifications of a wall. It should be pointed out that this terminology sometimes is confusing, especially when more than one wall is present in a wall group. When discussing chlamydospore walls, Griffiths (1974) suggested that the term layer should be avoided and that internally differentiated lamellae seen in the walls should be considered as "zones". Later, Berch (1986) suggested the use of "wall layers" instead of simply "walls". We agree with Morton (1988) in that until the real nature and the development of walls are better understood it would be wiser to postpone changes, maintaining the terminology for walls of arbuscular mycorrhizal spores as proposed by Walker (1983).

*Acaulospora morrowiae* (A... 983)

This isolate produces spores globose to subglobose, with a smooth surface, 90 to 120  $\mu\text{m}$  diameter, borne singly in the soil. The spores are light yellow in water and bright yellow to yellow brown when mature and mounted on PVLG. They have a composite wall divided in three groups and separable in broken spores (Figs. 3.1, 3.2). The first group (A) consists of an evanescent and a laminated wall. Group B is formed by a semi-rigid wall, and group C has two walls: one membranous, beaded wall and one, the innermost, amorphous (Fig. 3.2). This wall group reacts in Melzer's,

becoming purple. With TEM it was not possible to distinguish the wall structure completely due to fixation problems. When spores were fixed with glutaraldehyde in a microwave oven and later broken in liquid N<sub>2</sub> (Chapter II h), it was impossible to recover them for examination. When spores were fixed in osmium/24 hr (Chapter II c), only part of the wall structure (group A) was fixed. The other wall groups and the sporoplasm were lost. Thus, it was discernible only as a series of regularly distributed fibrillar layers of regular thickness with an arced, bow-shaped appearance (Fig. 3.3). This apparently corresponds to the laminated wall seen with LM. In some spores a distinct band with a different, amorphous organization was distinguished in the inside part of the wall (Fig. 3.3), and sometimes detached from the arced layers (Fig. 3.4). This might correspond to the semi-rigid wall of group B. In this case TEM did not provide enough information about all wall ultrastructure to make accurate comparisons with the walls as observed with the LM. It was interesting to find again wall fibrils with the arced appearance, one feature that now seems quite common among the arbuscular mycorrhizal species.

*Acaulospora scrobiculata* (ASCB 984)

This species forms subhyaline to light brown or darker brown, 100 to 220  $\mu\text{m}$ , globose, ornamented spores. Four layers were originally described with LM: an outer layer

pitted with regularly distributed depressions; two thin, hyaline intermediate layers, and one innermost layer which has a "beaded" appearance and turns deep red in Melzer's reagent (Trappe, 1977). The isolate observed here with the LM had the wall separated in two groups (Figs. 3.5, 3.6). Group A has a 4-6  $\mu\text{m}$  thick wall, classified as unit (Walker, 1983), which is ornamented with deep and round bottomed depressions. Group B is formed by three walls: the first is unit and less than 1  $\mu\text{m}$  thick. The second has the same thickness, is membranous and has a "beaded" appearance. The third, innermost, is membranous and turns deep red in Melzer's. Depending on the pressure given to the spore under the cover slip, the first wall in group B will or will not easily separate from the other walls in this group (Figs. 3.5, 3.6). However, in general the separation between these two walls is quite obvious and therefore the "beaded" nature of the innermost wall becomes clear, especially when stained with Melzer's. Depressions of the outer wall (group A) are easily seen at the LM level (Fig. 3.7) but with SEM their regular distribution is more evident (Figs. 3.8, 3.9). This unit outer wall observed with TEM is thick and composed of a series of layers (up to 20) with an arced, bow shaped appearance (Fig. 3.10). The inner layers are thinner than the outer layers, which suggests that the wall develops new layers from the sporoplasm outwardly. This illusory arrangement of fibrils follows the depressions

which gives quite a peculiar and unique aspect to the wall. The arced appearance disappears gradually towards the inner layers until no more are discernible (Fig. 3.10). When wall group B is being analyzed it becomes more difficult to correlate aspects of the wall observed with the LM with the structure seen at the TEM level. As mentioned above, with the LM the wall group B is formed by three walls: one unit and two membranous, one of which with a "beaded" appearance. At the TEM level it seems that the first wall of group B corresponds to a thin layer with a perpendicular (radial) arrangement of fibrils (Figs. 3.10 - 3.12) while the innermost wall seems to be probably represented by a thicker layer formed by amorphous material (Fig. 3.10). The aspect of these inner walls can change according the way the spore is sectioned. For example, in cross section the walls do not show a beaded appearance (Figs. 3.10, 3.14) while in a tangential section the walls in group B look beaded Fig. 3.13). In the same way, the fibrillar arrangement can be quite difference in appearance. The observations at the TEM demonstrated well the arrangement of the wall group A: e.g., the ornamented wall with pores and arced appearance. These fibrillar layers with bow-shaped fibrils (Figs. 3.10 - 3.15) are similar to those observed in the other *Acaulospora* species studied here and in many other arbuscular mycorrhizal fungi. Another feature of this spore, the presence of a wall with a perpendicular arrangement of

fibrils of regular periodicity, has been reported in *A. laevis* (Mosse, 1970c), and in *Scutellospora nigra* (Old et al., 1973). Mosse (1970c) called it a "tripartite membrane" and suggested that it may be common in spores where inner and outer walls are easily separated. This has apparently been confirmed with *A. scrobiculata* where the wall with regular periodicity of fibrils may correspond to the place where wall groups A and B separates. The reason and function of this wall is not clear and apparently it has been found only in arbuscular mycorrhizal spores. Another interesting observation was the consistent presence of bacteria inside the depressions of the outer wall (Fig. 3.16). However, different from what occurs in other species, this outer wall does not seem to be degraded by the bacteria since the arrangement of wall fibrils follows the contour of each cavity (Figs. 3.15, 3.16). The hollows might provide an appropriate environment for their development and survival.

*Glomus* sp. (L... 906)

This isolate forms spores globose to subglobose, 60-150  $\mu\text{m}$  diameter. With LM, spore surfaces appear smooth or slightly roughened (Figs. 3.17, 3.18). The walls are in one group in which an inner and an outer wall can be distinguished in young spores (Figs. 3.17, 3.18). Mature spores usually lose the outer wall and leave only the inner,

laminated wall. With SEM it is possible to distinguish much bacteria attached to the spore surface (Figs. 3.19, 3.20). With TEM the two walls, in one group, as occurs in most species of *Glomus* (Morton and Benny, 1990), are also perfectly distinguishable (Figs. 3.21, 3.23).

The inner wall has two layers which are especially apparent in young spores. There is a dark, electron dense layer of consistent thickness, with two or three sublayers newly synthesized from the sporoplasm, which is of the same density (Fig. 3.22). In mature spores these sublayers are no longer distinguishable (Fig. 3.23). In addition, there is a multilayered fibrillar zone in which the inner part has parallel fibrils that appear to become gradually arched or outwardly helicoidal (Fig. 3.23). During maturation, the number of layers with the arched pattern increases to twenty or more and the outer layers are thicker ( $0.6\ \mu\text{m}$ ) than the inner ones ( $0.3\ \mu\text{m}$ ), suggesting that the wall develops from inside to outside.

The outer wall consists of a layer of parallel fibrils immersed in amorphous material (Fig. 3.21) separated from the inner wall by an electron dense, thin but well defined band (Fig. 3.22). This band has been suggested in other arbuscular mycorrhizal fungi to be formed of sporopollenin (Gripiollo and Bonfante-Fasolo, 1984). The outer wall initially represents more than 30% of the wall thickness. However, it later becomes thinner, and finally completely

disappears. This is the evanescent wall observed through the LM.

Ultrastructurally it seems that the fibrillar arrangement of inner and outer walls are different. In the outer wall fibrils appear in a parallel arrangement while in the inner wall, fibrils are disposed in an arced or helicoidal organization which is evident as bow-shaped lines in the wall. As discussed before, this artifact is formed when stacked layers with straight fibrils, regularly arranged in parallel, are rotated at a small angle with respect to the previous one. Bonfante-Fasolo and Vian (1984), Bonfante-Fasolo and Grippiolo (1984), and Gibson (1985) have shown spore walls of other arbuscular mycorrhizal fungi which have this arced organization and this seems to be a characteristic unique to this group of fungi. There are indications that chitin is the major element of both the outer and the inner wall and that the arrangement of fibrils is responsible for the differences in appearance (Bonfante-Fasolo, 1988). Bonfante-Fasolo (1988) reported that in *Gl. versiforme* an electron dense line is the natural starting point of the arced layers and that this line is not present in *Gl. macrocarpum*. In the case of this isolate, it seems that the layers with helicoidal appearance originate from the electron dense layer which is in close contact with the plasma membrane. Apparently material from

the cytoplasm is incorporated into the first wall layers (Fig. 3.32, arrow).

This isolate (L...906) resembles *Gl. etunicatum*; however, it differs in color and wall thickness. Earlier studies with *Gl. etunicatum* (Gibson, 1985) did not reveal a wall with arced appearance as it was observed in this isolate, but this could be the result of fixation problems.

Many bacteria were found present on and in the outer wall (Figs. 3.26 - 3.29). They apparently have an important role in wall degradation. Its evident that bacteria may start colonizing solid walls (Fig. 3.26) and can degrade them until reaching a point where wall materials have been gradually substituted by mucilaginous products of degradation (Figs. 3.26 - 3.29). Interesting to notice that such organisms were mainly localized in the outer wall, never crossing the dense line that separates the outer wall from the inner wall. This may enforce the hypothesis that this line is formed by sporopollenin, the most resistant polymer found in nature.

*Glomus* sp. (L... 312)

This isolate forms yellow, globose spores in the soil (average 120  $\mu$ m in diameter). The spore has two walls in one group (Figs. 3.30, 3.31). The outer wall is evanescent and has a roughed surface (Fig. 3.32); at higher magnification in the TEM it seems to be formed by parallel

fibrils embedded in an amorphous matrix (Fig. 3.33). The inner wall is formed by a series of layers of apparently arced fibrils (Figs. 3.34, 3.35), as seen in *Glomus* sp. (L...906) and other arbuscular mycorrhizal species. There is a distinct band linking inner wall and sporoplasm (Fig. 3.35, arrow), but zones of discrete wall formation where not seen. This may correspond to an inner, membranous wall sometimes observed in young spores (Fig. 3.30). In contrast to the isolate previously discussed (L...906), inner and outer walls of this spore are not separated by a marked electron dense zone. There is instead a very thin electron dense line apparent in some spores (Fig. 3.34).

*Glomus claroides* (LCRD 698)

The average size of the spores observed in this species varied from 100 to 140  $\mu\text{m}$  in diameter. This species was originally described as having one or two walls in one group, the outer being laminated (Schenck and Smith, 1982). However, the isolate observed here showed a different wall pattern. There are really three walls, in one group. The outer wall is thin, evanescent and consequently may not be present in mature spores (Figs. 3.36 - 3.38). The second wall is well delimited and laminated, although the laminae sometimes are not easily distinguishable (Figs. 3.37, 3.38). The innermost wall is very thin and membranous (Fig. 3.38). With TEM the laminated wall has a quite interesting aspect.

When spores were fixed with Karnovsky in a microwave oven, broken in liquid N<sub>2</sub> and postfixed with osmium (see Chapter II h), there were many open spaces in the wall as if it had been stretched (Fig. 3.39). When spores were fixed in 3% potassium permanganate (Chapter II e), the wall showed a roughened aspect with apparently parallel fibrils immersed in a dense, amorphous matrix (Fig. 3.40). The outer wall was lost most of the time, probably during the fixation process, but the small pieces that remained were consistently colonized by bacteria (Fig. 3.41).

Glomus sp. (L... 925)

The spores of this isolate have two walls: the outer is evanescent, thick and sloughing at maturity (Figs. 3.42 - 3.44). This is particularly evident when spores are observed with the SEM (Fig. 3.44). At the TEM level this wall appears to be composed of parallel fibrils distributed in an amorphous matrix (Fig. 3.45). The inner wall is laminated and, as in many other *Glomus* species, appears formed by a series of layers with an arced fibrillar arrangement (Figs. 3.45, 3.46). The arrangement of the spore wall in this species looks ultrastructurally similar to the wall of the isolate L...906 and other *Glomus* species in that there are also two walls, the outer evanescent and the inner persistent and laminated. This isolate and isolate L...906 also have in common a layer of electron

dense material separating the outer from the inner wall. However, with LM and TEM the outer, evanescent wall appear slightly different when micrographs are compared (Figs. 3.23, 3.45). Further studies will be necessary to confirm if they are isolates of the same species. It should be considered that even when the type (= classification of the wall) and the localization of walls are the same on different species, chemical composition, distribution of components and arrangement of fibrils might be different, suggesting diverse developmental processes.

*Glomus intraradices* (LITC 208)

This species is a very peculiar fungus because it produces most of its yellow to brown spores inside the root, different from the great majority of arbuscular mycorrhizal species which produce their spores outside the root. Although other species, such as *Gl. aggregatum* Schenck & Smith emend. Koske, *Gl. manihotis* Howeler, Sieverding & Schenck, *Gl. clarum*, *Gl. diaphanum*, *Gl. heterosporum* Smith & Schenck, and *Gl. microaggregatum* Koske, Gemma & Olexia have also been reported as producing spores inside the root (Morton, 1988), *Gl. intraradices* continues to be the most typical and characteristic among these fungi.

With LM the spore wall of *Gl. intraradices* is formed by an outer evanescent wall, and one, two or more inner laminated walls (Schenck and Smith, 1982). Laminations

sometimes are not so evident (Fig. 3.47). With SEM it is possible to see much debris attached to the spore surface (Fig. 3.48), a situation commonly found in soil borne spores. At the TEM level the wall structure looks different from all others examined in this study. At the beginning, when the spore is young, there is a thick wall with many layers, where only the outermost layer is distinct from the inner layers (Fig. 3.49). Later these inner layers start separating, and the outer layer becomes more evident (Fig. 3.50). The outer layer in this wall is always thicker and sometimes stains darker than the inner ones (Fig. 3.51). Apparently, new inner layers are produced from material coming from the sporoplasm and the separation of layers continues during the maturation process. Very often bacteria invade and degrade the outer, evanescent wall (Figs. 3.52 - 3.54). Differences in wall arrangement as observed in this species, depends on spore age. Thus, if a young spore is found, all layers are together (Fig. 3.49); later the layers separate and mature spores will have a series of separated layers which gives the appearance of laminations (Figs. 3.53 - 3.55). Observing only mature spores of *Gl. intraradices*, Gibson (1985) mentioned that the laminated wall consisted of several layers. The series of laminae could also be considered independent walls. In some cases, for example, there are separated groups of walls (up to 6) with a different number (1, 2, 5) of layers (Fig.

3.55). This gives rise to diverse interpretations about the wall structure and because of that, it is appropriate to look at a high number of spores before making any decision regarding the spore wall. Two characters were more evident in *Gl. intraradices*. First, the fibrils are not arranged in the apparent arcuate pattern as seen in many other arbuscular mycorrhizal fungi with laminated walls. Instead, it seems that the fibrils are in parallel but without a clear organization. Little detail of fibrillar arrangement in spores of *Gl. intraradices* has been reported (Gibson, 1985). Second, the laminations seen at the LM level are produced by a high number of layers or walls with a varied degree of aggregation between each other, depending on spore age. Morton and Benny (1990) observed that the inner laminated wall formed last in this species but they could not determine temporal progression of the outer layers with the LM. A peculiar characteristic of the laminated wall, as originally described (Walker, 1983), is that it begins as a single layer and becomes thicker with age as a consequence of production of new laminae. This has been confirmed with other species (Mosse, 1970c; Sward, 1981a). We agree with Mosse's (1986) interpretation that it is "...improbable that the apparent laminations seen by LM correspond to the layers of arced fibrils seen by TEM..." as suggested by Bonfante-Fasolo et al. (1985). The fact that *Gl. intraradices*, which

is described as having laminated wall does not show an arced appearance with TEM enforces this view.

*Gigaspora albida* (LABD 927)

This species is characterized by formation of white spores with a light yellow-greenish pigment, globose to subglobose, and 260 - 370  $\mu\text{m}$  diameter. Its spore wall was originally described as being formed by an outer, thin, smooth wall and two or three but occasionally 4 to 5 inner, inseparable walls (Schenck and Smith, 1982). At the LM level those inseparable walls give the appearance of laminations (Figs. 3.56 - 3.58); because of this character the species resembles, and has been confused, with *Gi. margarita*. They are slightly different in color, the former being greenish, the latter yellowish, but spore color has been shown to be highly variable (Morton, 1988). The size of the spores in both species also differs only slightly and evidence indicates that spore diameter should not be considered a strong character for species identification (Morton, 1988). However, as will be discussed in the next chapter, germination processes in these two species are different, which further separates them as independent taxa. Description of spore wall ultrastructure of *Gi. albida* proved to be a difficult task. This occurred mainly because spores at different stages of development were observed and consequently different wall patterns were found.

Nevertheless, this also provided opportunity for studying the development of the wall in this species. With the SEM invaginations of the wall can be distinguished (Fig. 3.59, arrow). As will be discussed later, this seems to be a common feature in these spores. On smaller, presumably young spores, the wall can be divided in four parts, or four walls (Fig. 3.60). One outer and easily detachable, electron dense wall, two inner walls, each of different aspect, thickness and pigmentation, seem not to be easily separable, and finally, there is one innermost electron dense wall that is slightly less dense than the outer wall.

When spores were fixed with Karnovsky plus permanganate (Chapter II d), the outer wall appeared very dark and electron-dense (Fig. 3.60). There was a thin dark line separating this outer wall from the inner walls. The first inner wall shows fibrils with parallel (Fig. 3.62), perpendicular (Fig. 3.63), or sometimes bent appearance. Below those and forming the third wall, there is the thickest part of the wall which seems to be composed by amorphous material (Figs. 3.64, 3.65). Inside, forming the fourth wall, are two bands of electron dense material. The innermost is thicker and is in contact with the sporoplasm. New sporoplasmic material appears to be gradually added to the wall and it become thicker in some points (Fig. 3.64). When mature spores are observed, it is possible to verify that the thick, third wall becomes layered as if laminations

were being produced (Fig. 3.65). The wall grows also towards the cytoplasm, forming mound-like structures or projections inside the cytoplasm. This material is layered with light and electron-dense zones (Figs. 3.66 - 3.68). In older or more mature spores the layers in the third wall are more evident and striations can be easily distinguished (Figs. 3.66 - 3.69). Later they become stronger, producing the laminated aspect of the wall as observed with the LM (Fig. 3.69).

The factors which produce those laminations are not clear. It can be a physiological process related with age or it can be related with cyclic events during the spore life. Another hypothesis is that the spore germinates when mature; if it does not find a suitable environment the germ tube degenerates and the spore reenters in dormancy. Each time this occurs the spore may form a new wall or wall layer to protect itself. Thus, spores with many laminations would represent many repetitions of that event. In his ultrastructural work with *Gi. margarita*, Sward (1981a) showed the same wall structure found in the isolate studied here. He interpreted the wall as being formed by four main layers and provided information about their chemical composition. The outer wall, or the first layer, was negative for chitin and there were indications of presence of other polysaccharides, proteins and lipids. The second layer, with striations of regular periodicity also had

negative results for chitin and was positive for lipids. Sward (1981a) proposed that the striations are in fact rods of lipid. Similar striate layers were also reported in *Scutellospora gregaria* (Gibson, 1985). The thicker, third layer was positive for chitin. Possibly the wall biochemical reactions could be tested again for confirmation of its composition. When enzymes were used to digest the wall there were no remarkable differences in the wall ultrastructure, as expected of walls formed by chitin and other easily degraded polysaccharides. This may indicate that the concentration of the enzyme was not appropriate, that time was not enough to allow degradation of such thick wall or that there is some compound in the wall which are not degraded by the enzyme used. Here again comes the hypothesis that sporopollenin can be a common component of spore walls in arbuscular mycorrhizal fungi. Major differences in structural components are hardly found in taxa with similarities in spore morphology (Bartnicki-Garcia, 1968).

A special feature in all *Glomus* spores observed here was the presence of an evanescent wall. Although not formed in spores of *Gigaspora* and *Scutellospora* species, this wall is quite common among representatives of *Glomus* and *Acaulospora* (Morton, 1988). The main characteristic of this wall is that it sloughs as a spore matures. Thus, in some cases presence of soil debris attached to the spore surface

can be misinterpreted as being an evanescent wall and only when some spores in a population show an intact wall it is possible to have it correctly identified (Morton, 1988). The outer wall of an arbuscular mycorrhizal spore, if covered or not by an outermost evanescent wall, probably protects the spore against dehydration and against the action of other soil microorganisms. As it has been suggested, it may be functionally analogous to the cuticle in higher plants (Tewari et al. 1982).

Most of the species observed here have their spores surrounded by bacteria. Attempts were made to identify the most common species. Seven of them were isolated; however, the results of the identification were not conclusive because the library of fatty acid profiles utilized was more related with phytopathogenic bacteria. Further analyses will be necessary to identify those species and compare the results with that obtained in other studies.

It seems that the environment in which the bacteria develop around the spore should be suitable for their growth and proliferation, and it may become a special niche where, in most cases, the prime food source is the wall polysaccharides. The bacteria may produce enzymes capable of degrading chitin and other wall constituents. Colonization of arbuscular mycorrhizal spores by chitin-decomposing microorganisms has been reported elsewhere (Ames, 1989; Ames et al., 1989; Mayo et al. 1986).

It is interesting to observe that the bacteria were found present in walls of many different species and isolates and growing under diverse environmental conditions such as pot cultures and aeroponic cultures. This may indicate that what is necessary for the bacteria is the spore wall itself and not the place where the fungi occur. Maybe these organisms are developing a mutualistic symbiosis where the fungus provides protection and is the food source while the bacteria helps in the activation of determined physiological processes. Ames et al. (1989) suggest that there may be a parasitic relationship occurring between the two organisms. They based their conclusions on studies with *Gl. macrocarpum*. Most of the actinomycetes associated with spores and hypha of this fungus were chitin decomposing and some were photographed etched into the spore wall. There is perhaps the possibility that the bacteria act indirectly on the spores by inactivating inhibitory compounds present in the environment or that they may act directly, promoting the potentialities of the spores to overthrow the inhibition (Tommerup, 1985). Other evidences have indicated the presence of self-inhibitors in the spore of some Glomales species (Daniels and Trappe, 1980). Thus, it is possible that soil microorganisms such as the spore associated bacteria and actinomycetes eliminate these self-inhibitors, therefore triggering spore germination. There are reports of stimulation of germination of arbuscular mycorrhizal

spores by the presence of bacteria or even by the presence of some volatile compound they produce (Mayo et al. 1986; Mugnier and Mosse, 1987; Tommerup, 1985; Tylka et al. 1991). Conversely, there are only a few indications that the presence of some bacteria may delay or inhibit germination of arbuscular mycorrhizal fungi (Paulitz and Linderman, 1989; Tylka et al., 1991). A recent report mentions that application of rhizosphere bacteria increased arbuscular mycorrhization. This increase was suppose to be the result of the direct effects of the bacteria on the fungus or to an influence on root physiology (von Alten et al., 1991). Limited knowledge about spore associated bacteria, however, does not allow us to draw conclusions about the role they may have in the life cycle of the arbuscular mycorrhizal fungi.

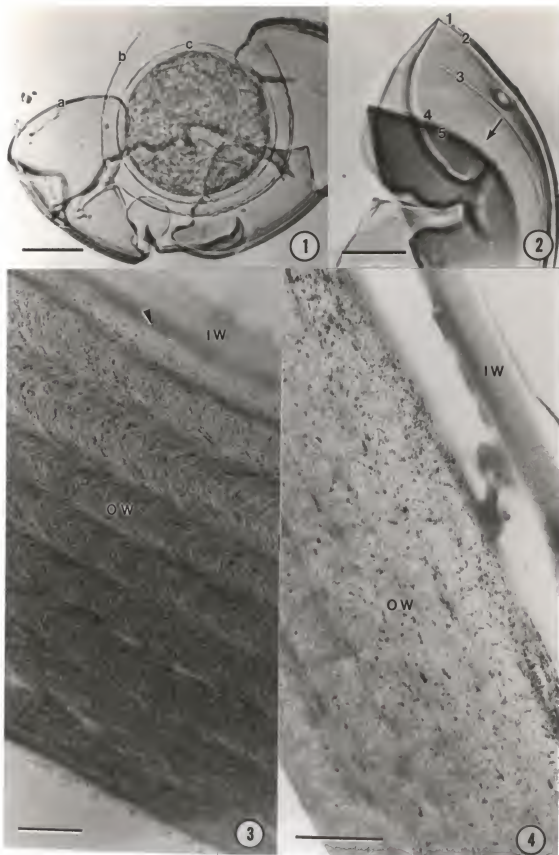
Figs. 3.1 - 3.4 - Light and electron micrographs of spores of *Acaulospora* sp. (A...983).

Figure 3.1 - LM of broken spore showing three wall groups: group A (a); group B (b); group C (c). (Bar= 25  $\mu$ m).

Figure 3.2 - Detail of walls in each wall group, as seen with LM. Group A= walls 1 and 2 (1,2); group B= wall 3 (3); group C= walls 4 and 5 (4,5). Note beaded wall (4, arrow). (Bar= 25  $\mu$ m).

Figure 3.3 - TEM of wall 2, group A (OW) and first inner wall or wall 3, group B (IW). Note apparent line where walls separate (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 3.4 - TEM of wall 2 (OW) separated from the first inner wall, or wall 3 (IW). (Bar= 0.5  $\mu$ m).



Figs. 3.5 - 3.12 - Light, scanning, and electron micrographs of spores of *Acaulospora scrobiculata* (ASCB 984).

Figure 3.5 - Light microscope micrograph of spore segment showing wall group A (a) and wall group B (b). (Bar= 25  $\mu$ m).

Figure 3.6 - Spore segment showing four walls, in two groups. Wall group A: wall 1 (1), and wall group B: walls 2, 3, and 4 (2,3,4). Note beaded appearance of wall 3 (arrowed). (Bar= 25  $\mu$ m).

Figure 3.7 - Light micrograph of spore surface, showing pits (arrowhead). (Bar= 25  $\mu$ m).

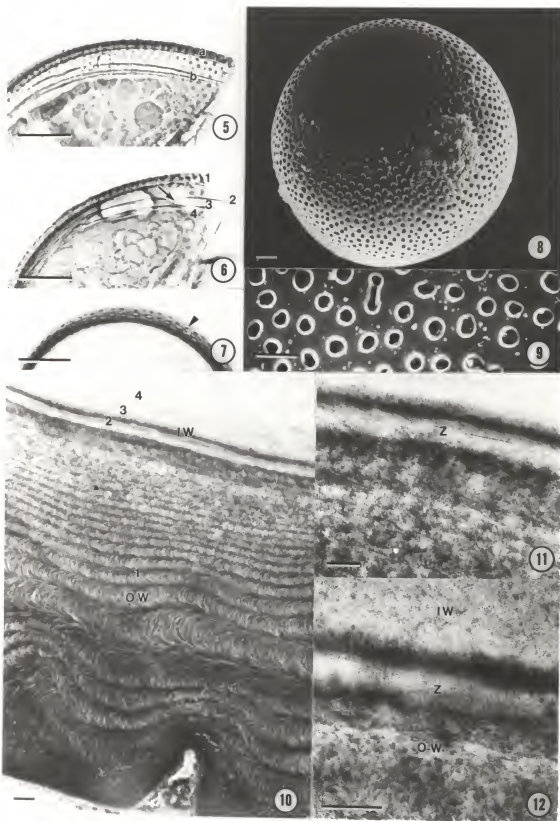
Figure 3.8 - Scanning electron micrograph of spore with scar (X) left by the sporogenous hypha. (Bar= 10  $\mu$ m)

Figure 3.9 - Detail of ornamented spore wall with pits. (Bar= 5  $\mu$ m).

Figure 3.10 - Spore wall as seen with TEM. Note wall group A with wall 1 (OW - 1), and wall group B with walls 2, 3, and 4 (IW - 2, 3, 4). (Bar= 5  $\mu$ m).

Figure 3.11 - Detail of wall 2, with radial arrangement of fibrils (Z). (Bar= 0.2  $\mu$ m).

Figure 3.12 - Higher magnification showing detail of wall 2 (Z) with fibrils apparently distributed radially, and separating wall group A (OW) from wall group B (IW). (Bar= 0.2  $\mu$ m).



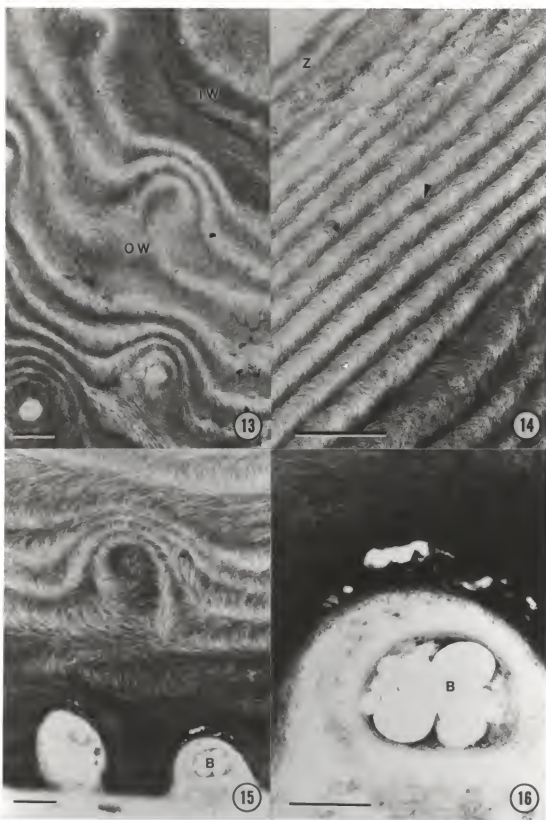
Figs. 3.13 - 3.16: Transmission electron micrographs of spore walls of *Acaulospora scrobiculata* (ASCB 984).

Figure 3.13 - Outer wall (OW) and inner wall (IW) which correspond to wall group A and wall group B. The arrangement of fibrils follows the contour of the outer, ornamented wall (arrowhead). Inner walls show beaded appearance (large arrow). (Bar= 1  $\mu$ m).

Figure 3.14 - Detail of spore walls showing outer wall with apparent bow-shaped fibrillar arrangement (arrowhead). (Bar= 1  $\mu$ m).

Figure 3.15 - Outer spore wall with depressions of the ornamentation filled with bacteria (B). (Bar= 1  $\mu$ m).

Figure 3.16 - Detail of one of the depressions of the ornamented wall filled with bacteria (B). Degradation of the wall by these bacteria is not evident. (Bar= 0.5  $\mu$ m).



Figs. 3.17 - 3.25: Light, scanning and electron micrographs of spores of *Glomus* sp. (L... 906).

Figure 3.17 - Light micrograph of segment of spore showing one wall group (a), and spore content with large lipid globule (L). (Bar= 25  $\mu$ m).

Figure 3.18 - Detail of spore wall at LM showing outer, evanescent wall (1) and inner, laminated wall (2), both in one group. Note laminations (arrow). (Bar= 20  $\mu$ m).

Figure 3.19 - Scanning electron micrograph of spore, showing spore surface covered by bacteria (B) and subtending hypha in evidence (S). (Bar= 25  $\mu$ m).

Figure 3.20 - Detail of spore surface covered by bacteria, as seen with the SEM (Bar= 5  $\mu$ m).

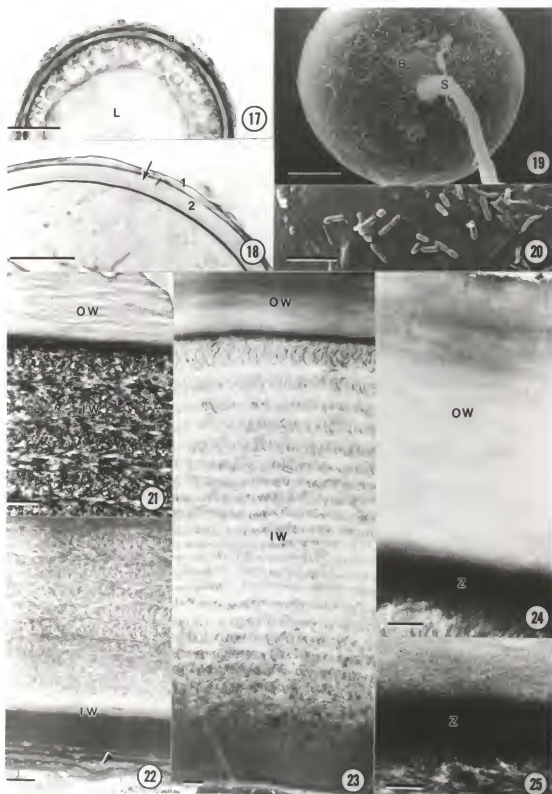
Figure 3.21 - Transmission electron micrograph of outer and inner wall. Outer wall (OW) with parallel arrangement of fibrils and inner wall (IW) with fibrils apparently arced. (Bar= 0.5  $\mu$ m).

Figure 3.22 - Detail of inner wall (IW) showing layers newly synthesized (arrowhead) and a multilayered fibrillar zone in which fibrils apparently become gradually arched outwardly. (Bar= 0.5  $\mu$ m).

Figure 3.23 - General view of inner and outer walls, showing large series of layers with apparently arced distribution of fibrils in the inner wall (IW) and of apparent amorphous material (OW). Note that new layers of inner wall, shown in Fig. 3.22 are no more distinguishable. (Bar= 0.5  $\mu$ m).

Figure 3.24 - Detail of outer wall (OW) with amorphous appearance. Note separation zone between outer and inner walls (Z). (Bar= 0.5  $\mu$ m).

Figure 3.25 - Higher magnification of the electron dense band (Z) which separates inner from outer wall. (Bar= 0.2  $\mu$ m).



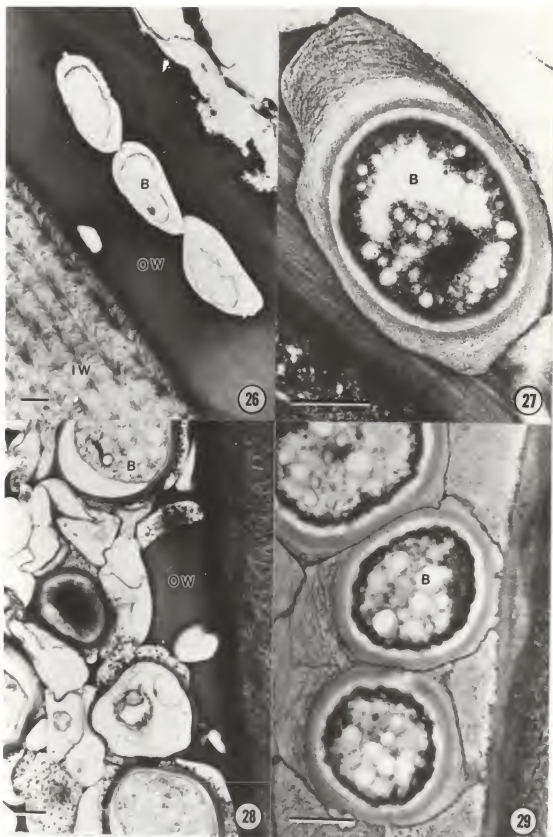
Figs. 3.26 - 3.29: Transmission electron micrographs of spore walls of *Glomus* sp. (L... 906).

Figure 3.26 - Bacteria (B) embedded in the outer wall (OW) of a spore (B). (Bar= 0.5 $\mu$ m).

Figure 3.27 - Detail of a bacteria (B) degrading the outer wall. (Bar= 0.5  $\mu$ m).

Figure 3.28 - Colony of bacteria (B) in a partially degraded outer wall. (Bar= 0.5  $\mu$ m).

Figure 3.29 - Colony of bacteria (B) in a matrix which replaces the outer wall of the spore. (Bar= 0.5 $\mu$ m).



Figs. 3.30 - 3.35: Light and transmission electron  
micrographs of *Glomus* sp. (L...312).

Figure 3.30 - General aspect of a spore with LM, showing subtending hypha (S) and wall group A with an outer, evanescent wall (1) and an inner, laminated wall (2). (Bar= 25 $\mu$ m).

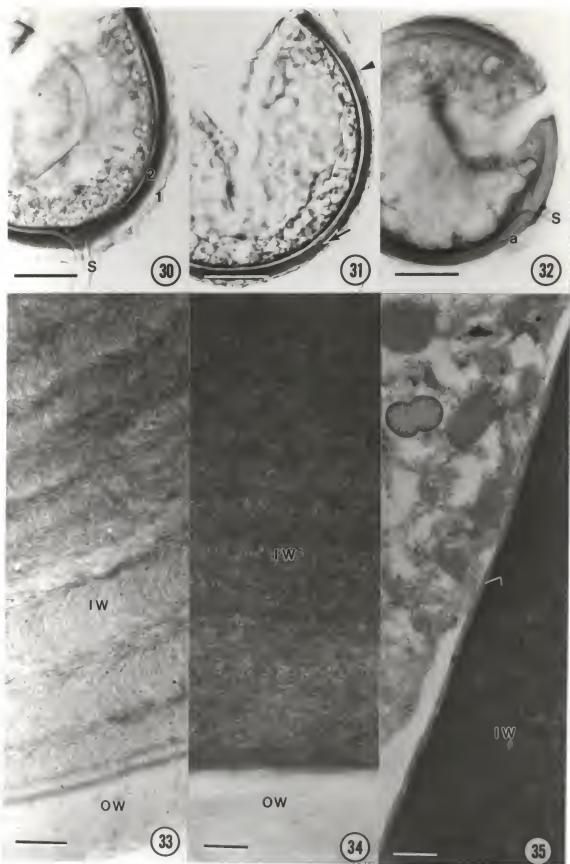
Figure 3.31 - Aspect of a broken spore at the LM level with evanescent wall almost indistinct (arrowhead). Note laminations of wall 2 (arrow). (Bar= 25  $\mu$ m).

Figure 3.32 - Aspect of an older spore with LM. Note subtending hypha (S) and wall (a). (Bar= 25 $\mu$ m).

Figure 3.33 - Wall structure at TEM level, showing outer wall (OW) and inner wall (IW) with layers of apparent arcuate fibrils. (Bar= 1  $\mu$ m).

Figure 3.34 - Outer wall (OW) and inner wall (IW), the latter showing apparently parallel fibrils dispersed in amorphous matrix. (Bar= 1  $\mu$ m).

Figure 3.35 - Detail of inner wall (IW) and a thin layer of a different wall material (arrowhead) which separates wall and sporoplasm. (Bar= 1  $\mu$ m).



Figs. 3.36 - 3.41: Light and electron micrographs of spores of *Glomus claroides* (LCRD 698).

Figure 3.36 - General view of spore with LM. Note three subtending hyphae (S). (Bar= 30  $\mu$ m).

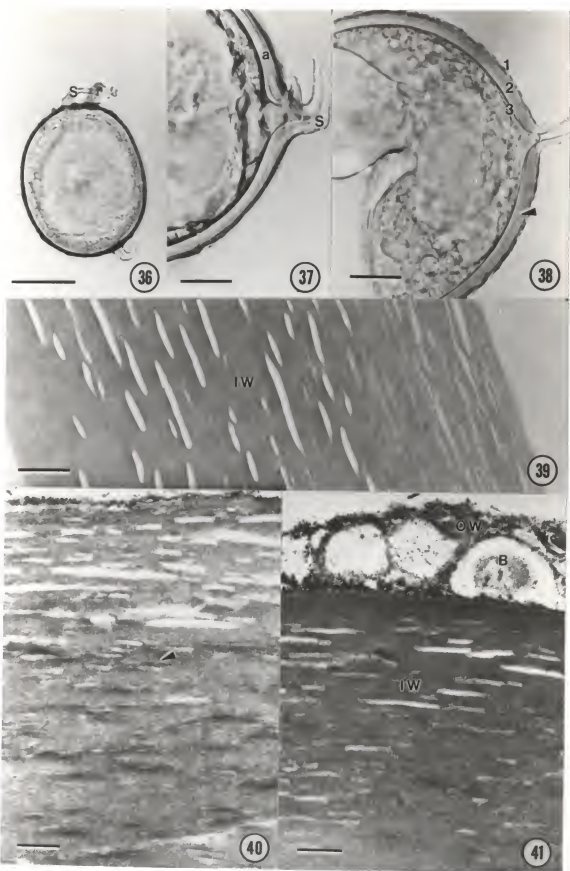
Figure 3.37 - Detail of spore showing walls in one group. Note subtending hypha (S). (Bar= 20  $\mu$ m).

Figure 3.38 - General aspect of broken spore at the LM showing three walls in one group. The outer is evanescent, the second is laminated (arrowhead), the third is membranous (1, 2, 3). (Bar= 20  $\mu$ m).

Figure 3.39 - Transmission electron micrograph of the laminated wall (IW)). Note stretched aspect of the spore wall fixed with Karnovsky in a microwave oven, broken in liquid N<sub>2</sub>, and postfixed in osmium. (Bar= 0.5  $\mu$ m).

Figure 3.40 - General aspect of laminated wall, fixed with 3% potassium permanganate. Note roughened aspect of wall and apparent parallel fibrils (arrowhead) immersed in an amorphous matrix. (Bar= 0.5  $\mu$ m).

Figure 3.41 - Detail of outer (OW) and inner (IW) walls. Note bacteria (B) degrading the outer, evanescent wall. (Bar= 0.5  $\mu$ m).



Figs. 3.42 - 3.46: Light, scanning and transmission electron micrographs of spores of *Glomus* sp. (L...925).

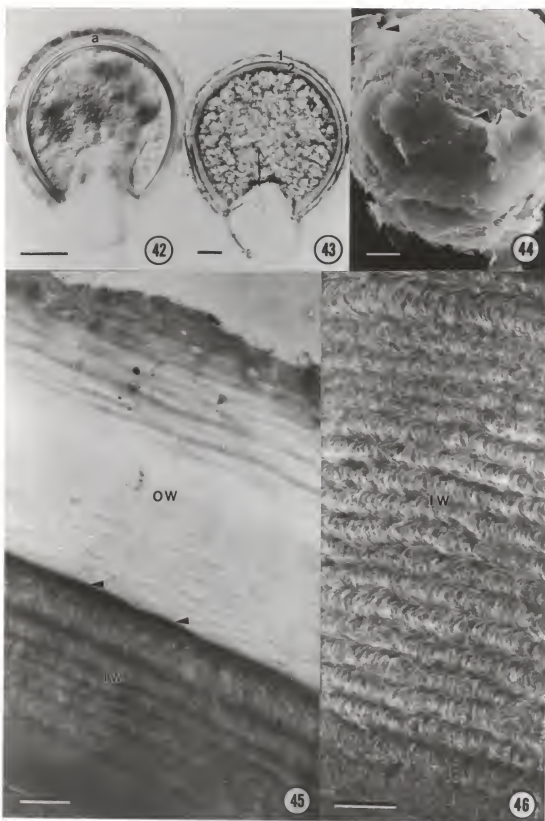
Figure 3.42 - General aspect of broken spore at the LM level in Nomarsky interference contrast. Note wall in one group (a). (Bar= 20  $\mu$ m).

Figure 3.43 - Aspect of broken spore as seen with LM. Outer wall evanescent (1), inner wall laminated (2), clearly differentiated. (Bar= 20  $\mu$ m).

Figure 3.44 - Scanning electron micrograph of spore showing sloughing of outer wall (arrowhead). (Bar= 50  $\mu$ m).

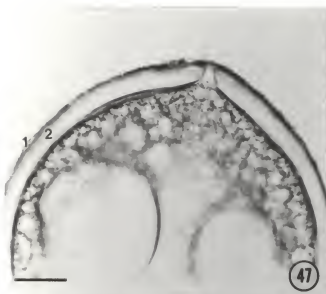
Figure 3.45 - Transmission electron micrograph of inner (IW) and outer (OW) walls. Note thin layer of electron dense material which separates the two walls (arrowheads). (Bar= 1  $\mu$ m).

Figure 3.46 - Detail of inner wall (IW) with apparent pattern of arcuate fibrils. (Bar= 1  $\mu$ m).



Figs. 3.47 - 3.51: Light, scanning and transmission electron micrographs of spores of *Glomus intraradices* (LITR 208).

- Figure 3.47 - Aspect of a broken spore showing wall structure and subtending hyphae scar (arrowhead) at the LM level. Wall in one group: outer wall evanescent (1), inner wall laminated (2). (Bar= 20  $\mu\text{m}$ ).
- Figure 3.48 - Spore inside a root segment as seen with SEM. Note subtending hypha (S). (Bar= 20  $\mu\text{m}$ ).
- Figure 3.49 - Laminations of inner wall (IW) in a young spore, as observed with TEM. (Bar= 0.5  $\mu\text{m}$ ).
- Figure 3.50 - Detail of inner, laminated wall showing more distinct laminations (arrowhead). (Bar= 0.5  $\mu\text{m}$ ).
- Figure 3.51 - Well developed laminations of inner wall in older spore. Note separation of outermost layer (arrow). (Bar= 0.5  $\mu\text{m}$ ).



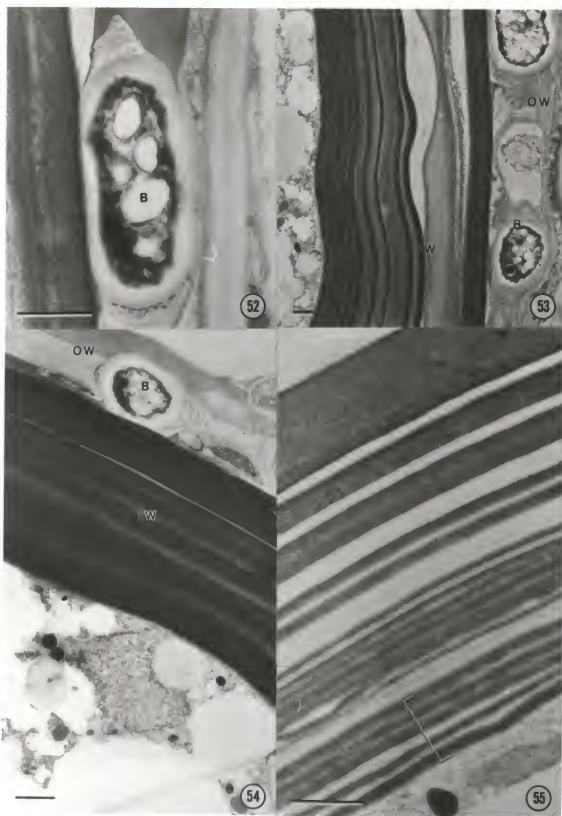
Figs. 3.52 - 3.55: Transmission electron micrographs of  
*Glomus intraradices* (LITR 208).

Figure 3.52 - Detail of a bacteria (B) degrading outer wall of a spore. (Bar= 0.5  $\mu$ m).

Figure 3.53 - Outer wall of a spore highly colonized by bacteria (B). Note laminations and separation of the inner wall layers (IW). (Bar= 0.5  $\mu$ m).

Figure 3.54 - Continuous separation of layers in the inner wall (IW) and colonization of outer wall (OW) by bacteria (B). (Bar= 0.5  $\mu$ m).

Figure 3.55 - Detail of inner wall in an older spore with a series of laminated walls (parenthesis). (Bar= 0.5  $\mu$ m).



Figs. 3.56 - 3.61: Light, scanning and transmission electron micrographs of spores of *Gigaspora albida* (GABD 927).

Figure 3.56 - Detail of spore wall with LM. Note wall in one group (a). (Bar= 20  $\mu$ m).

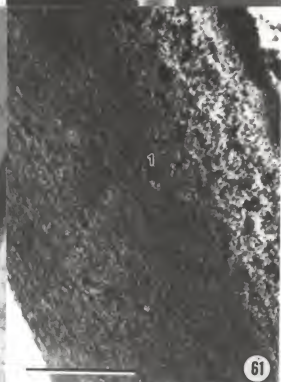
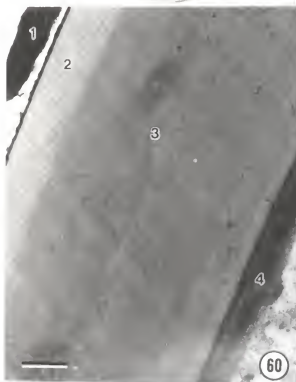
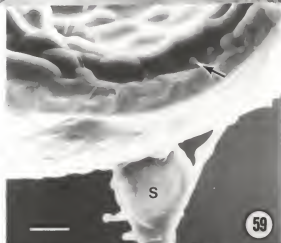
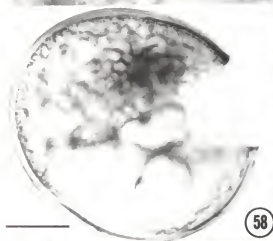
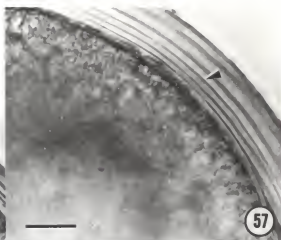
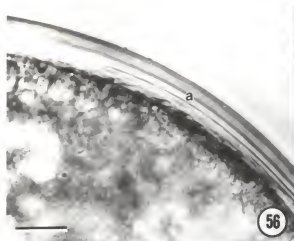
Figure 3.57 - Detail of a spore wall in older spore. Note higher number of laminations (arrowhead). (Bar= 20  $\mu$ m).

Figure 3.58 - General view of a broken spore with LM. (Bar= 100  $\mu$ m).

Figure 3.59 - Scanning electron micrograph of a broken spore, with projection of the wall towards the sporoplasm (arrow). Note sporogenous cell (S). (Bar= 20  $\mu$ m).

Figure 3.60 - Transmission electron micrograph of wall structure. Four walls (1, 2, 4, 4) are clearly distinct. (Bar= 0.5  $\mu$ m).

Figure 3.61 - Detail of outermost wall. (Bar= 0.5  $\mu$ m).



Figs. 3.62 - 3.65: Transmission electron micrograph of spore wall of *Gigaspora albida* (GABD 927).

Figure 3.62 - Detail of outermost wall (1) and first inner wall (2). Note wall 2 with apparent parallel arrangement of fibrils (lines). (Bar= 0.5  $\mu$ m).

Figure 3.63 - Observation of wall 2 at higher magnification (2). Note apparent perpendicular arrangement of fibrils (lines). (Bar= 0.5  $\mu$ m).

Figure 3.64 - Detail of walls 3 and 4 (3, 4). Observe increase in thickness of wall 4 (X). (Bar= 0.5  $\mu$ m).

Figure 3.65 - General aspect of wall 3 forming first laminations (arrowhead) and view of wall 4 with projections (mound shaped structures) towards the sporoplasm (X). (Bar= 0.5  $\mu$ m).



Figs. 3.66 - 3.69: Transmission electron micrographs of spore walls of *Gigaspora albida* (GABD 927) showing development of laminations and projections in walls 3 and 4.

Figure 3.66 - Initial formation of laminations (arrow) in the third wall (3) and increase in thickness (X) of the fourth wall (4). (Bar= 0.5  $\mu$ m).

Figure 3.67 - Development of mound shaped structures (X) by wall 4. Note layers of different electron density (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 3.68 - Detail of projection (X) formed by wall 4 showing layers of different electron density. (Bar= 0.5  $\mu$ m).

Figure 3.69 - Aspect of wall 3 in older spore showing laminations clearly developed (arrow). Note wall 2 with apparent perpendicular fibrils (2). (Bar= 0.5  $\mu$ m).



CHAPTER IV  
MORPHOLOGICAL AND ULTRASTRUCTURAL ASPECTS  
OF SPORES AND GERM TUBES

Introduction

Zygomycetes are a group of fungi characterized by formation of distinctive sexual spores, the zygospores. However, classification in all groups of Zygomycetes has been based largely on the anamorph (Benjamin, 1979), while zygospores, even though not used as the main character, have been also taxonomically useful.

The arbuscular mycorrhizal fungi are Zygomycetes which until recently were included among the Endogonaceae (Endogonales). This classification was recognized as artificial by many authors since the relationships of arbuscular mycorrhizal fungi with *Endogone* were based on superficial affinities of habit (sporocarpic) and morphology (Gerdemann and Trappe, 1974; Morton, 1988; Morton and Benny, 1990; Walker, 1987), but there was no clear evidence as how to change such an unnatural arrangement. Among the genera included earlier in the Endogonales, only *Endogone* had been reported to produce zygospores (Bucholtz, 1912; Gerdemann and Trappe, 1974; Kanouse, 1936; Thaxter, 1922). *Endogone* spp. are saprophytic fungi which may occasionally form

ectomycorrhizae (Bonfante-Fasolo and Scannerini, 1977); all other genera included earlier in Endogonales were symbionts, forming arbuscules in their host. Then, recently all arbuscular mycorrhizal forming fungi were separated in a distinct order, Glomales. This order was erected to include all soil-borne fungi forming arbuscules in obligate mutualistic symbiosis with plants. The new order Glomales was an attempt at a natural classification which grouped the arbuscular fungi based on patterns of ancestry (Morton and Benny, 1990). The criteria they used for characterizing genera were somatic and reproductive stages of known species, spore ontogeny, and the types of spore germination. This and previous studies demonstrate how important spore and hyphal characters are for understanding the arbuscular mycorrhizal fungi. Reproduction in Glomales was believed to be by asexual (chlamydospores) or parthenogenetic (azygospores) spores (Gerdemann and Trappe, 1974; Hall, 1984; Harley and Smith, 1983). However, it was later reported that isolates of *Gigaspora decipiens* were able to produce zygospores after conjugation of germ tubes from paired "azygospores" (Tommerup, 1988; Tommerup and Sivasithamparan, 1990). This special event was seen as a clear evidence that at least one genus of arbuscular mycorrhizal fungi (Morton, 1988) and by extension, Glomales, was correctly placed in Zygomycetes. Nonetheless, the formation of those supposed zygospores remains unclear,

and attempts to demonstrate any evidence of sexual reproduction through cytological studies among Glomales have failed. As reported by Schipper (1987), the search for the perfect stage in the Zygomycetes started soon after the discovery of two phases in the life cycle of *Syzygites megalocarpus* Ehrenb.: Fr. by Tulasne (1855). Since then, results in many cases have been disappointing, even after the pioneer work of Blakeslee (1904) who demonstrated heterothallism in Zygomycetes, and the discovery of pheromones which induce zygospore formation (Schipper, 1987). Studies of nuclear events revealing mitotic or meiotic processes, karyogamy, and formation of zygospores in arbuscular mycorrhizal fungi can also be included among those with "disappointing" results. Thus, since there is no evidence to the contrary, spores formed by most Glomales genera (excluding now the zygospores produced by *Gigaspora*) could be considered an anamorphic phase. In Zygomycetes the anamorph is represented by sporangiola, sporangiosporos, merosporangia, and chlamydospores. Spores formed by members of Glomales have been usually considered as chlamydospores, as in *Glomus* and *Sclerocystis* (Gerdemann and Trappe, 1974; Hall, 1984; Schenck and Pérez, 1990), or azygospores, as in *Acaulospora*, *Entrophospora*, *Gigaspora* and *Scutellospora* (Gerdemann and Trappe, 1974; Hall, 1984; Morton, 1988; Schenck and Pérez, 1990). Morton and Benny (1990), in their classification of Glomales families, considered not only the

spores of Glomaceae (*Glomus* and *Sclerocystis*) but also the propagules of Acaulosporaceae (*Acaulospora* and *Entrophospora*) to be chlamydospores. Gibson (1985) and Walker (1987) suggested that spores of *Acaulospora* and *Entrophospora* might be single-spored sporangia. Based on spore wall data, septal ultrastructure and mode of germination, Gibson (1985) suggested that the azygospores of *Gigaspora* may represent, in fact, zygozspores in which there is a reduction of one gametangium to form the sporogenous cell, while chlamydospores of *Glomus* may exemplify a further reduction to a single gametangium. Tommerup and Sivasithamparam (1990) proposed that spores of *Gigaspora* and *Glomus* are blastic conidia exhibiting rhexolytic secession and, therefore, they would be similar to the aleuriospore of some authors. A broad discussion about the use of the terms chlamydospore and azygospore for spores of arbuscular mycorrhizal fungi was also presented by Tommerup and Sivasithamparam (1990) who claim that since bi- and monogametangial teleomorphs have being found in *Gi. decipiens*, the term azygospore, denoting asexual spores, should not be used. They consider that once there is evidence for sexual reproduction, azygospore is an anomalous term, even for the other Zygomycetes.

An azygospore is a zygozspore-like spore which develops from a single gametangium without any evidence of sexual fusion (Benjamin, 1979). It is well established that

nuclear fusion is the confirmation of a sexual process (Blakeslee, 1904). However, in many Zygomycetes which produce zygospores nuclear fusion has not been described. In his studies with diverse Zygomycete taxa, Cutter (1942) was unable to find any indication of nuclear fusion or meiosis in some of these species although zygospores were produced. Sassen (1965) observed paired nuclei in the progametangia of a *Phycomyces* species; however, fusion of those nuclei was not established. Sward (1981b) recorded the presence of paired nuclei in spores of *Gi. margarita* but was not able to see mitotic spindles or any other indication of nuclear division. Nuclear fusion was never reported in any species of Endogonales or Glomales, although zygospore formation has been described in both orders (Gibson, 1985; Tommerup, 1988).

Azygospores are not frequently produced in Zygomycetes (Gooday et al., 1973). However, species exist which produce abundant obligate azygospores but not zygospores. Examples are *Mucor azygosporus* Benjamin, *M. bainieri* Mehrotra & Baijal, *M. tenuis* Bainier, *M. ardhlaengiktus* Mehrotra & Mehrotra, and *Absidia spinosa* Lendner var. *azygospora* Boedijn (Benjamin and Mehrotra, 1963; Mehrotra and Mehrotra, 1978; O'Donnell et al., 1977). Production of azygospores has been suggested to result from diverse circumstances such as strong sexual ability of the partners: by this hypothesis, when sexual reproduction is blocked, the latent

potency of one of the partners is expressed (Blakeslee, 1904); weak sexual potency of the partners with consequent prevention of zygospore formation (Ling Young, 1930), unfavorable cultural conditions during sexual reproduction (Schipper, 1973); imperfect hybridization (Schipper, 1975) or absence of interaction of fully active (+) and (-) factors (Schipper and Stalper, 1980).

Chlamydospore is a term used broadly to denote in general any asexual, thick walled spore. Griffiths (1974) discussed the origin, structure and function of chlamydospores in fungi and compared them with other spore types. His concept of chlamydospores states that they are produced within hyphae, do not constitute part of the regular life cycle and should therefore be considered as passive secondary spores. However, the term has been used also to denote spores whose function is primarily dissemination (Hughes, 1953). Griffiths (1974) considered that in Mucorales the term should be restricted to thick walled spores formed within the vegetative mycelium. Thus, azygospores, which are borne in specialized branches, would not be chlamydospores. Relative to zygomycete spores, Benjamin (1979) agrees with Griffiths concepts regarding the term chlamydospore. In his revision of Endogonaceae, Thaxter (1922) stressed the confusion centered around the concepts of chlamydospores and zygospores, indicating that they could be distinguished by looking at details of the

junction between the spores and the hyphae where they develop. As shown by Powell et al. (1981) in studies with *Gilbertella persicaria* (Eddy) Hesseltine, the septa of chlamydospores contain plasmodesmata which indicates a continuous interaction between the chlamydospore and the hypha. Cytoplasmic continuity through plasmodesmata was reported to occur between chlamydospores or azygospores and the hypha where they are formed and between zygozspores and their gametangia, in three orders of Zygomycetes: Mucorales (O'Donnell et al., 1977; Powell et al., 1981; Benny and Benjamin, 1991), Glomales (Gibson, 1985; Morton and Benny, 1990), and Endogonales (Gibson et al., 1986). However, secondary thickening occurs on both sides of the septum of an azygospore or a zygozspore, instead of only on the inner side as occurs in the chlamydospores (O'Donnell et al., 1977; Powell et al., 1981). Although chlamydospores and zygozspores have some characters in common, i. e. both are thick-walled spores presumably important for perennation, and during their development pass through extensive changes in cell wall and cytoplasm, it is clear that the ontogeny of these spores is different (Powell et al., 1981).

Grove (1976) reviewed the form and function of zygomycete spores and pointed out that even though most of the work in this area had been done with mucoraceous species, the knowledge about controls over activation and germination was still very limited. Siqueira et al. (1985)

reviewed the literature about spore ontogeny and morphology, mechanisms and factors affecting germination, and germ tubes of arbuscular mycorrhizal fungi. Subsequently, many reports have dealt with germination of spores in different species of Glomales (Azcón-Aguilar et al., 1986; Daniels and Trappe, 1980; El-Atrach et al., 1989; Gianinazzi-Pearson et al., 1989; Mayo et al., 1986; Safir et al., 1990; Tommerup, 1985; Tylka et al., 1991; Wilson et al., 1987). However, as mentioned before, there are few reports on ultrastructure of spores, their formation, development and germination in Glomales. The only ultrastructural studies available about spores, germination and germ tube growth of arbuscular mycorrhizal fungi are those by Mosse (1970a, 1970b, 1970c) on *Acaulospora laevis*, Old et al. (1973) on *Scutellospora nigra* (Redhead) Walker & Sanders, Sward (1981a, 1981b, 1981c) on *Gigaspora margarita*, and by Gibson (1985) on spores of some species of *Glomus*, *Gigaspora* and *Scutellospora*. Other ultrastructural reports are related with spore wall structure (Bonfante-Fasolo, 1982; Bonfante-Fasolo and Vian, 1984, 1989; Gibson et al., 1987), or with the anatomy of the endomycorrhizal interaction (Bonfante-Fasolo, 1982; Bonfante-Fasolo and Vian, 1989; Bonfante-Fasolo et al., 1990; Codignola et al., 1989; Garriock et al., 1989; Jabaji-Hare et al., 1990; Yawney and Schultz, 1990).

In describing changes which occur during the development of spores to the dormancy stage in *Acaulospora laevis*, Mosse (1970b) pointed out the presence of three unusual organelles inside the spores: pigment granules, large crystals and self duplicating bacteria-like organisms. The dormant spore was characterized as having very little cytoplasm, very large oil globules and large round bodies which were suppose to be a storage polysaccharide. Using a schematic drawing, Mosse (1970b) described the changes in cytoplasm and wall structure related to spore development.

Old et al. (1973), in studies with *Scutellospora nigra*, besides giving a detailed description of the spore wall, discussed the formation of peripheral compartments preceding germination. This type of germination was also described in *A. laevis* (Mosse, 1970a), *S. gilmorei* (Trappe & Gerd.) Walker & Sanders (Gerdemann and Trappe, 1974; Sward, 1978), and *S. aurigloba* (Hall) Walker & Sanders (Hall, 1977). Production of germ tubes without the formation of peripheral compartments was reported in *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe, *S. heterogama* (Nicol. & Gerd.) Walker & Sanders, and *S. calospora* (Nicol. & Gerd.) Walker & Sanders (Nicolson and Gerdemann, 1968). Later it was shown that the germination processes in species of *Acaulospora*, *Gigaspora*, and *Scutellospora* are different (Gibson, 1985; Morton and Benny, 1990; Sward, 1981b; Walker and Sanders, 1986). Although not describing in detail the

germination processes in the species he studied ultra-structurally, Gibson (1985) was able to show germ chambers in spores of *S. heterogama* and *S. pellucida*. The type of germination was one of the main characteristics used by Walker and Sanders (1986) to separate the genera *Gigaspora* and *Scutellospora*. Germination in *Gigaspora* species occurs by direct penetration of germ tubes through the spore wall, (Gibson, 1985; Nicolson and Gerdemann, 1968; Sward, 1981b, 1981c), while germination in species of *Scutellospora* occurs after formation of a germination shield in an inner wall group of membranous or coriaceous walls (Gibson, 1985; Morton and Benny, 1990; Walker and Sanders, 1986). Morton and Benny (1990) consider that *Gigaspora* is ancestral to *Scutellospora* and therefore should be technically (using cladistic analysis) grouped together. Nevertheless, they maintain both as separate genera until more information becomes available about the walls associated with the germination processes in these taxa.

Sward (1981a), describing the structure of spores of *Gi. margarita*, introduced two new terms, the "synthesizing" and the "nuclear" centers to characterize regions which contain different proportions of various spore organelles. Thus, the "synthesizing" center contained a large number of organelles associated with biosynthetic activity while the "nuclear" center, besides having some of the organelles found in the synthesizing center, as the name indicates

contained a large number of nuclei. It was noticed that two of the unusual organelles mentioned by Mosse (1970b), i. e. crystals and bacteria-like organisms, were also found in spores of *Gi. margarita* (Sward, 1981a). The onset of germination was shown to be remarkable by the activity and redistribution of the spore cytoplasm, accompanied by changes in wall structure (Sward, 1981b). Based on Mosse (1970b), Becker and Hall (1976) and Sward (1981b, 1981c) descriptions, Siqueira (1983) and Siqueira et al. (1985) presented a schematic representation of the germination process as it occurs in spores of *Gi. margarita*.

There are no ultrastructural studies on germination processes of *Entrophospora*, *Glomus*, and *Sclerocystis* species. However, germination in *Entrophospora* is suspected to be similar to that described for *Acaulospora* (Morton and Benny, 1990) where pre-germination compartments are formed. Germination of *Glomus* spores occurs by emergence of the germ tube through the subtending hypha (Gerdemann and Trappe, 1974; Mosse, 1959; Morton and Benny, 1990), although direct germination through the spore wall has also been shown in different species (Hall, 1977; Louis and Lim, 1988; Walker and Rhodes, 1981). Germination of *Sclerocystis* spores has never been described but it probably is similar to one of the processes in *Glomus* since they are closely related genera.

Many investigators have determined the multinucleate condition of spores, extramatrical and intramatrical mycelium, auxiliary cells, vesicles and arbuscules of arbuscular mycorrhizal fungi (Bonfante-Fasolo et al. 1981; Burggraaf and Beringer, 1987; Cooke et al., 1987; Mosse, 1970a; Scannerini and Bonfante-Fasolo, 1975; Sward, 1981b). However, the significance of the multinuclear state and the nature of the nuclei, if homokaryotic or heterokaryotic, needs to be determined.

Ultrastructural studies have shown that light microscopy is insufficient to reveal details of structures which are important for characterization of a taxonomic group. Therefore, ultrastructural data may provide information which can be useful in understanding phylogenies. Evolutionary relationships within the Zygomycetes are not well understood and the relationship of Glomales with the other groups in this class is still unclear. The main objectives of this investigation were to determine ultrastructural features which characterize the Glomales and compare these data with those from other Zygomycetes orders, especially Endogonales, and to better understand the position of the Glomales among the Zygomycetes. Attempts were made to observe any evidence of nuclear fusion or meiotic and mitotic divisions in spores, and hyphae of different arbuscular mycorrhizal species that

would provide information to clarify the true nature of the spores produced by Glomales.

### Materials and Methods

#### Source of Materials

Vegetative and reproductive structures from the following isolates of arbuscular mycorrhizal fungi were used in this study:

*Glomus* sp. (INVAM - L... 925)

*Glomus intraradices* (INVAM - LITR 208)

*Glomus* sp. (INVAM - L... 906)

*Gigaspora albida* (INVAM - GABD 927)

The fungal material was obtained from pot cultures of a sand-vermiculite mixture in association with bahia grass and selected as described in Chapter II.

#### Germination

Spores of L... 925 were selected, transferred to Petri dishes and left in the refrigerator (4°C) for few days. Some of them were attached to mycelium and later some others which were only with the hyphal attachment developed germ tubes.

Spores of L... 906 and LITR 208 were selected under a dissecting microscope, washed, superficially disinfected with 0.5% NaOCl (10% Chlorox) for 2 min, washed three times

in distilled sterilized water and placed in Petri dishes with 1% water agar + 0.01% yeast extract and incubated as mentioned below.

Presumed healthy spores of GABD 927 were selected, washed in water, superficially disinfected as described above or not disinfected, and allowed to germinate in Petri dishes with 1% water agar + thiamine at  $0.1 \text{ mg/l}^{-1}$ .

Plates of all isolates were incubated at room temperature ( $25^{\circ}\text{C}$ ) in the dark and germination was accessed every other day. Spores of *Glomus* were considered germinated when the germ tube was twice the size of the spore. Spores of *Gigaspora* were considered germinated as soon as a germ tube was apparent.

The spores of all isolates, along with their respective hyphae and germ tubes, were processed as reported below.

Material from all isolates, consisting of germinated spores, germ tubes and developed mycelium were fixed in 7.2% glutaraldehyde (Chapter IIa) or Karnovsky's (Chapter IIb) and embedded as described earlier. To avoid loss, the material was embedded in 2% agarose before fixation and strips of this agarose containing spores and germ tubes were then processed. Sectioning and staining of blocked materials were performed as reported in Chapter II.

### Light Microscopy (LM)

Selected spores with or without attachments, germ tubes and hyphae were mounted on a microscope slide either in water, Melzer's or PVLG (polyvinyl alcohol-lactic acid-glycerol - Koske and Tessier, 1983) and observed with a Nikon compound light microscope equipped with Nomarski differential-interference contrast optics.

### Scanning Electron Microscopy (SEM)

Germinating spores of GABD 927 were prepared for examination in a Hitachi S-456 scanning electron microscope as recorded in Chapter II.

### Transmission Electron Microscopy (TEM)

All material was prepared for TEM as depicted in Chapter II. Details of the processes employed for each isolate are as follows:

*Glomus* sp. (L... 925) - samples of spores, germ tubes and attached mycelium were fixed in Karnovsky, modified as in Chapter II (b), or fixed in glutaraldehyde in a microwave oven, broken in liquid N<sub>2</sub>, and post-fixed with 1% osmium tetroxide as described in Chapter II (h).

*Glomus* sp. (L... 906) and *Gl. intraradices* (LITR 208) - the spores and attached material were fixed with glutaraldehyde in a microwave oven, broken in liquid N<sub>2</sub>, and

post-fixed with 1% osmium tetroxide as recorded in Chapter II (h).

*Gigaspora albida* (GABD 927) - the material was fixed in Karnovsky as in Chapter II (b) or fixed in Karnovsky followed by 0.5% potassium permanganate, as reported in Chapter II (d).

After fixation the material was embedded, thin sectioned, collected on one-hole copper grids, post-stained and observed in a JEOL-100CX electron microscope, as detailed in Chapter II. To enhance the appearance of nuclear structures inside the hyphae, some grids were stained with uranyl acetate plus 1% methanol/30 min at 60°C, washed with 50% methanol, washed twice in distilled water, stained with lead citrate/5 min, washed again with distilled water and later observed in a JEOL-100CX electron microscope.

### Results and Discussion

In general the spore germination rate was low (in some cases less than 10%) and many of the germinating spores were surrounded by bacteria. This indicates that the germination potential of the spores was not affected by the bacterial colonies which developed even around the superficially sterilized spores. *Gigaspora* spores germinated more easily and faster when compared with spores of the *Glomus* isolates. The ultrastructure of mature spores of an undescribed

species of *Glomus* (L... 925) and of *Gl. intraradices*, and the ultrastructure of mature and germinating spores of *Glomus* sp. (L... 906) and *Gi. albida* will be discussed.

*Glomus* sp. (L... 925)

Spores of this isolate are borne on short or long hyphal segments not differentiated from the mycelium (Figs. 4.1 - 4.3). The spores have one large to many small lipid globules easily observed with LM (Figs. 4.2, 4.3). Sometimes the spore would germinate, developing a new hypha opposite the point of hyphal attachment (Fig. 4.3), a phenomenon reported also in spores of *Gl. clarum* (Louis and Lim, 1988). The mycelium can be septate or not, and nuclei are regularly distributed along the hyphae (Figs. 4.4 - 4.7). Interhyphal proliferation of older hypha is apparently a common event in this isolate. In the material observed, it was evident that the "outer" hyphal wall was colonized by microorganisms (Figs. 4.6, 4.7). Proliferation of new hypha inside old ones in other species of arbuscular mycorrhizal fungi have been reported in *Glomus aggregatum* Schenck & Smith emend. Koske (Koske, 1985) and *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe (Wu and Chen, 1985).

Fixation of spores of *Glomus* sp. (L...925) did not provide satisfactory results. However, general observations about the spore structure can be discussed. Apparently the

distribution of sporoplasmic organelles is similar to what has been reported in other arbuscular mycorrhizal spores in which there is a large amount of lipid globules and vacuoles. Nuclei and other organelles are distributed between them (Figs. 4.8, 4.9). Polyphosphate granules and electron dense, irregularly defined granules were also observed, the latter usually inside the vacuoles (Fig. 4.8). Elongate or rounded mitochondria and variable-sized lipid droplets were generally found in the nuclear region (Figs. 4.10, 4.11). Bacteria-like organelles (BLOs) were commonly seen dispersed throughout the sporoplasm (Fig. 4.11). Nuclei were isolated, in pairs or in larger groups (Figs. 4.11 - 4.15). Some of these nuclei presented clusters of chromatin near the nuclear envelope (Figs. 4.12, 4.15). Two of the paired nuclei (Fig. 4.13) were connected by a bridge where only one nucleolus was evident. Unfortunately, it was not possible to know if these nuclei were dividing or fusing when the spore was fixed.

#### *Glomus intraradices* (LITC 208)

Attempts to obtain germinated spores of this isolate failed. Therefore, only spore structure will be discussed. Many of the spores observed, presumably in their mature stages since the wall structure was already formed by many layers, contained various small and a few large vacuoles and many lipid globules, some of them coalescing, which was

evident with both LM and TEM (Figs. 4.16, 4.17). The usual sporoplasmic organelles were visibly compressed between the vacuoles and the spore wall, forming a peripheral layer (Figs. 4.18 - 4.20). Mosse (1970b) mentioned that in spores of *Acaulospora laevis* this peripheral layer is not established, but the cytoplasm is compressed into interstices between the oil vacuoles. A nuclear and an organizing center, as described by Sward (1981a) in spores of *Gigaspora margarita*, were not distinguished in the spores of this isolate. Nuclei were distributed along the proximities of the wall in the same way as mitochondria, ribosomes, small vacuoles, membrane bound polyphosphate granules and lipid globules (Figs. 4.18 - 4.20). Some of the vacuoles were apparently empty; others had dense materials which could be storage elements (Figs. 4.21, 4.22). In many cases the mitochondria were surrounding the nuclei (Figs. 4.21 - 4.24) but a connection was not observed between the nuclear envelope and the mitochondrial membrane, as seen in spores of *Glomus* sp. (L... 906). No evidence of nuclear division was observed but, in one of the nuclei, some lines which could be microtubules were noticed (Fig. 4.23). It has been suggested that nuclear division occurs in the spore before or during germination (Burggraaf and Beringer, 1987; Sward, 1981b). An indication of nuclear division may be the common occurrence of nuclei in pairs and the large number of nuclei present in each spore. However,

there is no evidence showing when and how this division occurs. The fact that smaller paired nuclei are common in spores and larger unpaired nuclei are consistent in the hyphae, suggest that some nuclear phenomena occurs during germination.

Glomus sp. (L... 906)

Spores of this isolate are borne terminally or intercalary on a hypha and can be abundantly produced either in pot culture, associated with bahia grass or in aeroponic culture in association with sweet potato. The young spore is filled with small lipid globules which later become larger and sometimes coalesce, forming one big globule. With LM, spores appear to have granular contents and many lipid droplets are evident (Figs. 4.25, 4.26). A general view of the spore with TEM reveals that it has many irregularly shaped vacuoles, and oil and lipid globules, with sporoplasmic organelles dispersed between them (Fig. 4.27). A higher number of these organelles are seen in the proximity of the spore wall (Fig. 4.27). Numerous electron dense granules are dispersed in the sporoplasm or sometimes inside the vacuoles (Figs. 4.27, 4.29). They were thought to be glycogen granules (Sward, 1981c). Membrane bound polyphosphate granules are also dispersed in the sporoplasm (Figs. 4.27, 4.28). Apparently similar structures were interpreted by Mosse (1970c) to be "pigment granules" and

some of them were shown to be incorporated into the pigmented outer wall of *Acaulospora laevis*. There were no indications that this occurs in the spore wall of *Gl. etunicatum*. Sward (1981a) suggested that these dense granules, also present in spores of *Gi. margarita*, are some form of lipoprotein storage body. Gibson (1985) interpreted them as the polyphosphate granules described by White and Brown (1979). As will be shown later, apparently these dense bodies which are common in spores, may coalesce forming large structures which are stored inside vacuoles in the germ tube (Figs. 4.35, 4.36). A self duplicating BLO first reported by Mosse (1970b) as an unusual organelle present in spores of *A. laevis* was regularly found in the spores (Fig. 4.28). They are irregularly round and in many instances divide by fission. These BLO's have a dense outer layer, a plasmalemma-like membrane and dense particles, as was noticed by Mosse (1970a) and Sward (1980b). The function of the BLOs is unknown. They have been found, however, also in germ tubes and mycelial hyphae (Figs. 4.42 - 4.44) of this species, in germ tubes of *Gi. margarita* (Sward, 1981c), and in hyphal coils and arbuscules of mycorrhizal roots of tomato, onion and white clover (Sward, unpublished data). They seem to complete their life cycle inside the fungus and apparently depend on it for nutrients. Attempts to isolate these organelles for further identification were unsuccessful (Sward, 1981a). Presumably

the occurrence of such organelles cannot be described any more as unusual since they have been found in other arbuscular mycorrhizal fungi so far studied ultra-structurally (Mosse, 1970b; Sward, 1981a), and their presence seems to be unique to this group of fungi.

Very often nuclei are in pairs (Figs. 4.29 - 4.32), as reported also in spores of *Gi. margarita* (Sward, 1981c), or in groups of four or six (Figs. 4.33). Nuclei have a granular content with dispersed chromatin, and a nucleolus may be clearly distinguished in some (Figs. 4.28 - 4.30). Mitochondria are long or slightly round and are always around the nuclei or in their vicinity (Figs. 4.28, 4.30, 4.32, 4.34). Connection between the nuclear membrane and the outer mitochondrial membrane (Figs. 4.32, 4.34) has not been previously reported. The significance of this association is not clear. Numerous small lipid droplets and vacuoles are clustered around the nuclei (Figs. 4.28, 4.32).

Modifications in wall structure and distribution of cytoplasmic organelles occurs during spore germination, as expected. Changes in wall structure from the spores to hypha, and from its extraradical to intraradical phase have been discussed in detail (Bonfante-Fasolo, 1988; Bonfante-Fasolo and Gianinazzi-Pearson, 1986; Bonfante-Fasolo et al., 1987). Hyphal walls are thinner than spore walls (Figs. 4.35 - 4.41), have a roughed surface and are formed by apparently amorphous material, although Bonfante-Fasolo

(1988) have described hyphal walls of arbuscular mycorrhizal fungi as of fibrillar texture. The cytoplasm contains large nuclei and numerous vacuoles with electron dense, globose bodies (Figs. 4.35, 4.36). An electron dense line is observed between the wall and the plasma membrane and it may be formed by addition of new wall material (Fig. 4.38, arrow). At higher magnification, the wall appears to have two distinct layers (Fig. 4.41). This may correspond to the structure of an older hypha where primary and secondary walls have been formed. A general view of the hypha shows that lipid globules, frequent in the spores, are no longer present. They are probably used as energy source for germ tube formation. When a spore germinates its reserves are depleted and the germ tube will end up as an assimilative hypha (Koske, 1981). A highly active cytoplasm is evidenced by the presence of many elongated mitochondria, and microtubules (Figs. 4.37, 4.38), a cytoskeletal element associated with mechanochemical forces for hyphal tip growth (Heath, 1990). Cytoplasmic microtubules are suppose to be involved also in the various types of nuclear movements cited for fungal cells (Heath, 1981a). Mitochondria and ribosomes probably provide energy for hyphal elongation. Lomasomes can be observed in proximity to the hyphal wall, isolated or in groups of small vesicles enclosed by a membrane (Fig. 4.39). There is a vacuolation zone where at least three types of vacuoles are distinguished: a) a few

small, apparently empty vacuoles (Figs. 4.36, 4.39); b) many large vacuoles, with one or occasionally more dark, irregularly delimited electron dense bodies and electron dense irregular amorphous granules dispersed around the vacuolar membrane (Figs. 4.39, 4.40), which are probably polyphosphate granules as discussed below; and c) groups of small vacuoles with double membranes inside larger vacuoles (Fig. 4.41). The BLOs found in the spores are also present, many in the process of binary fission (Figs. 4.42 - 4.44). The nuclei distributed along the hyphae are large, filling the cytoplasm (Fig. 4.36). No evidence of meiotic or mitotic processes were found. Looking at inter- and intracellular hypha of a *Glomus* species associated with roots of *Allium porrum*, Bonfante-Fasolo et al. (1981) observed that nuclei were unevenly distributed, had varied shape, and were usually in a peripheral position, near the cell wall. The multinucleate condition was evident in all the structures they studied (hyphae, vesicles and arbuscules).

*Gigaspora albida* (GABD 927)

Spores germinate easily after a few days of incubation, forming long, ramified mycelium. In many cases more than one germ tube is produced (Figs. 4.45, 4.46) and when this occurs the older germ tubes start forming septa distally (Fig. 4.47). The apex of these germ tubes become empty and

the part separated by septa soon degenerate (Fig. 4.49). The hyphal network of arbuscular mycorrhizal fungi is highly specialized both in structure and function (Friesse and Allen, 1991). In the mycelium developed on cultural media it was observed that hyphal segments start to degenerate, and segments with and without living cytoplasm are separated by septa (Figs. 4.47 - 4.51). It has been reported that mycorrhizal hypha develop septa as they lose their contents and function (Mosse and Hayman, 1980). Those septa seem completely closed but evidence of plasmodesmata was observed (Fig. 4.50, arrow). It is possible that more wall material had been added to the wall. This probably represents a defense mechanism since it is necessary for the hyphae to separate the parts which are degenerating, without prejudice to the rest of the hypha. Gibson (1985) showed that the septa in *Glomus*, *Sclerocystis* and *Endogone* do possess plasmodesmata; however, he was unable to study septa of *Gigaspora* species. Benny and Benjamin (1991) discussed the types of septa which occur in Zygomycetes, indicating that the multiperforate septa are characteristic of the orders Mucorales, Endogonales and Glomales which appear to be related. Another characteristic observed on the mycelium of *Gi. albida* was, as mentioned for other arbuscular mycorrhizal fungi (Koske, 1985; Wu and Chen, 1985), the formation of new hypha inside old ones (not shown). It is not known why the new hypha grows inside the old segments,

but perhaps it may be using some of the metabolites originated from cell degradation.

In general, the germ tubes emerge near the sporogenous cell (Figs. 4.52, 4.54) but they can also be produced in other parts of the spore. A close examination of the sporogenous cell with the SEM has revealed, in some spores, the presence of a hyphal segment originating from it (Fig. 4.53). Nicolson and Gerdemann (1968) reported the occurrence of sporogenous cells with a slender hypha extending from it to the base of some spores of *Scutellospora heterogama* (as *Endogone heterogama*), suggesting that they represent small suspensors. Spain et al. (1989) mentioned similar structures in *Gigaspora ramisporophora* Spain, Sieverding & Schenck, and suggested they might be vestigial sporophore branches. This segment could hypothetically be considered also as an aborted antheridium. If spores can be formed from them as observed by Spain et al. (1989), we agree that they could be just ramifications of the sporophorus cell. Nevertheless, more conclusive evidence is needed to define such structures. Multiple germination of *Gigaspora* spores, as observed here (Fig. 4.52), has been discussed (Koske, 1981; Mosse, 1959). This phenomenon could provide various benefits to the fungus, such as longer persistence of propagules in the soil when conditions are not favorable for mycorrhizal formation, and reduction in the vulnerability of the spores to other soil microorganisms, especially

mycophagous species (Griffins, 1972; Koske, 1981). Koske (1981) suggested that multiple germination could be a common occurrence among arbuscular mycorrhizal species. This would provide a higher chance of survival under various soil conditions.

The hyphal wall formed during germ tube emergence is thin and later, when the mycelium is well developed, it becomes thicker. The transformations that the wall undergoes both in morphology and composition during the life cycle of an arbuscular mycorrhizal fungi have been discussed (Bonfante-Fasolo, 1982, 1987, 1988; Bonfante-Fasolo et al., 1987). Those transformations occur after the spore germinates, passing through the extramatrical phase of the hypha in the soil to the intramatrical phase inside the roots. In the spore, the wall organization is more complex and elaborate. When the spore germinates there is a simplification of the wall structure although it is still thick and fibrillar in texture (Bonfante-Fasolo, 1988). During the intraradical phase the wall undergoes new changes in structure until it becomes much thinner, loses its fibrillar texture and becomes amorphous in structure (Bonfante-Fasolo, 1982; Bonfante-Fasolo et al., 1987).

The spore germinates directly through the wall (Fig. 4.54), a characteristic of *Gigaspora* species. Formation of germ tubes is a complex mechanism, with evident changes related to wall structure. The spore wall produces

protuberances into the sporoplasm (Figs. 4.55 - 4.57). These structures have been shown in spores of *Gi. margarita* (Sward, 1981b; Gibson 1985). Such protuberances will originate later in what has been described as the "germinal" wall (Spain et al., 1989), with mound shaped structures arising from the innermost wall of the spore (Figs. 4.56, 4.57). These wart-like structures are normally present in the spore wall of mature spores, independent of its germination condition. Gibson (1985) did not work with germinating spores and showed the same structures in *Gi. margarita*. Walker and Sanders (1986) have shown formation of a ring of warts on the interior of the laminated walls of *Gi. gigantea* prior to germination, assuming this characteristic is common to all *Gigaspora* species. A close examination shows that the innermost wall has layers of different electron density which are well differentiated in the mound shaped structures (Fig. 4.57). Apparently, it is between these structures that the germ tubes will be formed (Fig. 4.58). At the beginning the wall starts separating between the second and the third layers (from inside to outside), and unidentified material, whose origin and nature could not be determined, fills the space resulting from the separation of these layers (Figs. 4.58 - 4.61). If this material is of sporoplasmic origin, how then does it cross the existing wall? It could also be material originating from degradation of the wall which seems to be gradually

dissolved. In this case, an enzymatic rather than mechanical process would be responsible for germ tube emergence through the spore wall, as suggested also by Sward (1981b). Endogenous enzymes capable of splitting cell wall polymers are indispensable in many morphogenetic processes (Bartnicki-Garcia, 1968). Cytochemical methods need to be employed in order to determine the real nature of this material.

Formation of a new wall is evident in the part which is in direct contact with the wall that is being degraded (Figs. 4.59 - 4.62). This method of wall development was described by Bartnicki-Garcia (1968) as the "de novo" formation of a vegetative wall under the spore wall and it is apparently characteristic of fungi with chitosan-chitin walls such as Zygomycetes. It is interesting to note that the width of the inner layer, which is in contact with the sporoplasm remains apparently constant, while the outer parts of the walls are being dissolved and a tiny layer surrounded by new wall can be observed (Figs. 4.59 - 4.61). It is possible that the mound shaped structures are providing more strength to the wall which protect the spore contents while it is being penetrated by the germ tube. In the later stages, the germ tube continues to penetrate the spore wall (Fig. 4.62) until reaching a point where it will be outside forming a germ tube (Fig. 4.63). During this process, it can be observed that apparently there is an

increase in vacuolization, and lipid globules are no more present. There is also a complete new layer formed "de novo" around the germ tube. Outside the spore, the germ tube wall has two layers of different electron density (Fig. 4.63). The method of germ tube formation described here is quite different from that which was shown for *Gi. margarita* (Sward, 1981b) and *Gi. gigantea* (Nicolson and Gerdemann, 1968; Walker and Sanders, 1986) and for *Scutellospora* species, where germ shields are formed (Gibson, 1985; Walker and Sanders, 1986). The difference is the presence, in the spores studied here, of a wall layer separating the germ tube initial from the sporoplasm (Figs. 4.58 - 4.62). In *Gi. margarita* the development of the germ tube initial is followed by the intrusion of sporoplasmic contents from the germination region (Sward, 1981c). The method of germination reported here slightly resembles the one described by Mosse (1970c) for *A. laevis*, although it can not be considered the same since peripheral compartments were not defined. Perhaps the germination process described here could be considered an intermediate form between the germination processes of other *Gigaspora* species (direct penetration without a wall layer separating the germ tube initial from the sporoplasm) and of *Scutellospora* species (with formation of germ shields). The fact that good fixation and embedding of spores were not obtained was one

more barrier against a better understanding of the process of germ tube emergence in *Gi. albida*.

Hyphal development after germination shows a wall comparatively thinner in relation to the spore wall (Figs. 4.64 - 4.69). The wall has two layers, the inner thicker and electron dense, the outer thinner and sometimes with adherent particles (Figs. 4.65 - 4.68). A general view of these hyphae shows an uneven distribution of cytoplasmic organelles (Fig. 4.65). The hyphae are highly vacuolated, with mitochondria, polyphosphate granules, nuclei and other organelles seen in all of its extensions (Figs. 4.64 - 4.70). The vacuoles are of various sizes and shapes and may or may not have apparent contents. Sometimes they seem to be enclosing cytoplasmic structures such as electron dense globules, myelin figures, and polyphosphate granules (Figs. 4.65 - 4.67). However, differing from that found in hyphae of other arbuscular mycorrhizal species, they apparently do not accumulate the electron dense material reported here in hyphae of *Glomus* and in *Acaulospora laevis* (Mosse, 1970b). Mitochondria are found isolated or in groups throughout the hyphae and in some parts they seem to be the dominant organelle (Figs. 4.67, 4.68). These mitochondria in general are round shaped but may be elongated, especially near nuclei. There are many polyphosphate granules, bounded or not by a membrane (Figs. 4.64 - 4.70). The nuclei have different shapes and sizes but in general are large and can

be localized near the wall or occupying all hyphal width (Figs. 4.64, 4.66, 4.69 - 4.77). The nuclear envelope and the nucleolus were or were not well delimited in some spores, but the nuclear content was unclear probably due to fixation problems. Many nuclei were elongated, as if they were undergoing division (Figs. 4.72 - 4.77). In some instances, different structures not clearly identified, were seen in the nuclei (Figs. 4.73, 4.75, 4.76). The electron dense bands adjacent to the nuclear membrane (Fig. 4.73) could be remnants of the spindle pole bodies, also called nuclear associated organelles (NAOs) (see Beakes, 1981 and Heath 1981a, 1981b). In some Mucorales NAOs are associated with the inner nuclear membrane (Bland and Lunney, 1975). NAOs are probably implicated in major cellular processes such as nuclear division, nuclear movement and spore delimitation, and they are involved with both mitosis and meiosis (Heath, 1981b). However, their real function is not known (McKerracher and Heath, 1985). In one case it was possible to distinguish vaguely the NAOs and microtubules associated with division (Figs. 4.75, 4.76, arrows). Fungal spindles are small and intranuclear, and very often a population of cytoplasmic microtubules is found at the poles of mitotic nuclei (Heath, 1981a). In most Zygomycetes the nuclear envelope remains complete during the division process and the NAOs are truly intranuclear (Beakes, 1981). Here again, the fixation and staining processes proved to be

inadequate to provide good enhancement of nuclear structures. Many of the fixation processes used for TEM are known to produce artifacts or fail to preserve special structures (Beakes, 1981). There is a recognized heterogeneity in NAOs morphology in Zygomycetes (Heath, 1981b), and it is apparent that the forms of nuclear division that take place in Glomales have not been clearly identified. In another case, it was surprising to see a clear line in the central region of a nucleus, separating it in two parts (Fig. 4.77). Lines similar to microtubules were also seen, although not so clearly, crossing perpendicularly this nucleus. Some dark areas distributed in the nucleus could be interpreted as condensed chromatin. Chromatin condensation occurs normally during mitosis in most fungi (Heath, 1980). Some of the other features observed in these nuclei are similar with those reported in other Zygomycetes, especially Mucorales, where during mitosis chromosomes are not recognizable, the nucleolus is persistent and the NAOs are attached to the inner membrane of the nuclear envelope (Bland and Lunney, 1975; Franke and Reau, 1973; McCully and Robinow, 1973).

It seems useful to compare some of the features reported here with features pointed out by previous researchers. This could provide some more clues for better understanding Glomales and its relationships with other Zygomycete groups. Only one of the unusual organelles

discussed by Mosse (1970b, 1970c) was also present in the material studied, the BLOs. The large crystals reported in spores of *Acaulospora laevis* (Mosse, 1970b, 1970c) and *Gigaspora margarita* (Sward 1981a) were not found in any of the isolates studied. The pigment granules reported by Mosse (1970c) probably are different from what was seen in the spores observed here, which resemble most polyphosphate granules, as suggested by Gibson (1985). It is well known that the extramatrical hypha of arbuscular mycorrhizal fungi absorb phosphate (Thomson et al., 1990) and translocate it to the host plant. The absorption of phosphate results in a rapid conversion to nucleotide and in sugar phosphate, with the process followed by accumulation as polyphosphates (Ling-Lee et al., 1975). Polyphosphates are found in the fungus both as granules and as soluble polyphosphate (Martin et al., 1983). Polyphosphate in fact constitutes the primary phosphorus reserve for many fungi (Beever and Burnes, 1980); in arbuscular mycorrhiza it represents 16 to 40% of the total phosphorus in the fungus, although orthophosphates can also be stored (Gianinazzi-Pearson and Gianinazzi, 1986). The polyphosphate is accumulated in the vacuole forming metachromatic inclusions which at the TEM level are seen as electron-dense vacuolar granules. In this way, enclosed in the vacuoles, the phosphorus does not interfere with the cellular metabolism (Gianinazzi-Pearson and Gianinazzi, 1986). Tinker (1975) suggested that these

polyphosphate containing-vacuoles are carried by the streaming cytoplasm and continuously load and unload their contents, in such a way that a balance in the concentration of phosphorus can be maintained in the cytoplasm. Later, within the internal mycelium, the polyphosphate disappears from the vacuoles (Cappacio and Callow, 1982), indicating it has been transferred to the host.

Relative to the nuclear condition of spores and hyphae, there were indications that some division process was occurring although this could not be proven. In Zygomycetes, vegetative nuclear divisions are definitely mitotic (Beakes, 1981). In describing the onset of germination in *Gi. margarita*, Sward (1981b) said "... the nuclei may have divided because their number increased, and condensed chromatin and paired nuclei were commonly observed." Paired nuclei in the spores and elongated nuclei in the hyphae were also observed in this study. Elongated nuclei, or those with blebs, may correspond to anaphase stages (McCulley and Robinow, 1973) or can result from the flow of the cytoplasmic streaming (Beakes, 1981). It is well known that mitosis takes many forms and exhibits a wide range of variation. Heath (1980) discussed the significance of variations on fungal mitosis and mentioned that some species of Mucorales have been carefully examined but no convincing evidence for the presence of kinetochore microtubules were found. He suspects that Mucorales may

have the most primitive type of eukaryotic division because of the simplicity of the mitotic process. Thus, in this group during mitosis the spindle develops from entirely intranuclear NAOs, the nuclear envelope is intact, and the nucleolus persists and divides by simple constriction (Heath, 1980). These observations can be useful to enforce the view that mitotic processes normally occur in the spore of arbuscular mycorrhizal fungi but very often are difficult to observe. In order to continue looking for any evidence of fusion or division processes, spores will need to be fixed periodically at successive stages, while better methods for fixation, staining and embedding of nuclear structures must be developed before division processes can be observed properly at the ultrastructural level. It has been recognized that extensive serial sectioning is necessary for obtaining a complete picture of the nucleus and its NAOs (Beakes, 1981). The time taken to undergo mitosis varies greatly; some species complete the process in 5 min, other may need more than two hr (Heath, 1981a). In fungi, nuclear divisions are often very rapid (Beakes, 1981). How often and in which lapse of time does mitosis occurs in arbuscular mycorrhizal fungi? And what about meiosis? Studies of meiosis are not common, particularly in Zygomycetes (Beakes, 1981). In Glomales there is no proof that it occurs. Without evidence of meiosis such as

synaptonemal complexes, the anamorphic or teleomorphic nature of *Glomales* spores will remain a mystery.

Development of spores in *Glomus* and *Gigaspora* was shown to be distinct. Most authors recognize the spores of *Glomus* species as chlamydospores (Gerdemann and Trappe, 1974; Morton and Benny, 1990; Schenck and Pérez, 1990). Tommerup and Sivasithamparam (1990), however, suggest that these chlamydospores are blastic conidia, close to aleuroconidia (see Kendrick, 1985). They claim that these spores do not have a chlamydosporic type of ontogeny with cytoplasmic streaming still apparent in the subtending hypha when spores are mature. Griffiths (1974) mentioned that the protoplasm of the chlamydospore and adjacent hypha is continuous and indicated that this characteristic is not a factor against the chlamydosporic nature of a spore. However, the concept of chlamydospores given by Griffins (1974) defined them as perennation structures rather than as spores primarily involved with dissemination. Morton (1988) considered *Glomus* spores as chlamydospores but indicated that they could represent an intermediate form between chlamydospores and unispored sporangiola. Some Zygomycetes produce sporangiola, which are small sporangia containing one to many sporangiospores. Benjamin (1979) pointed out that the term "conidia" should not be used because it does not have a distinct meaning (it means dust). Moreover, he emphasized that while formation of endogenous chlamydospores is common

in Zygomycetes, development of truly exogenous spores i.e., unispored sporangiola, is rare. Again the discussion and doubts about the identity of the spores formed by *Glomus* species will remain unsolved until more knowledge is acquired.

Regarding *Gigaspora* spores, it seems that they will continue to be considered azygospores, unless evidence of their sexual nature is found. It should be mentioned, however, that they were recently considered as aleuroconidia (Tommerup and Sivasithamparam, 1990). The thin outer wall which encloses the thick laminated wall in many *Gigaspora* and *Scutellospora* species suggests a sporangial wall (Gibson, 1985; Morton, 1988; Walker, 1987). Much more study will be necessary to disclose the secret of spore formation in Glomales and their true sexual nature.

Figs. 4.1 - 4.7: Light micrographs of *Glomus* sp.  
(L...925).

Figure 4.1 - Spore borne from a single subtending hypha (S).  
(Bar= 20  $\mu$ m).

Figure 4.2 - General view of spore with large lipid globule  
(l). Note septum (arrowhead). (Bar= 20  $\mu$ m).

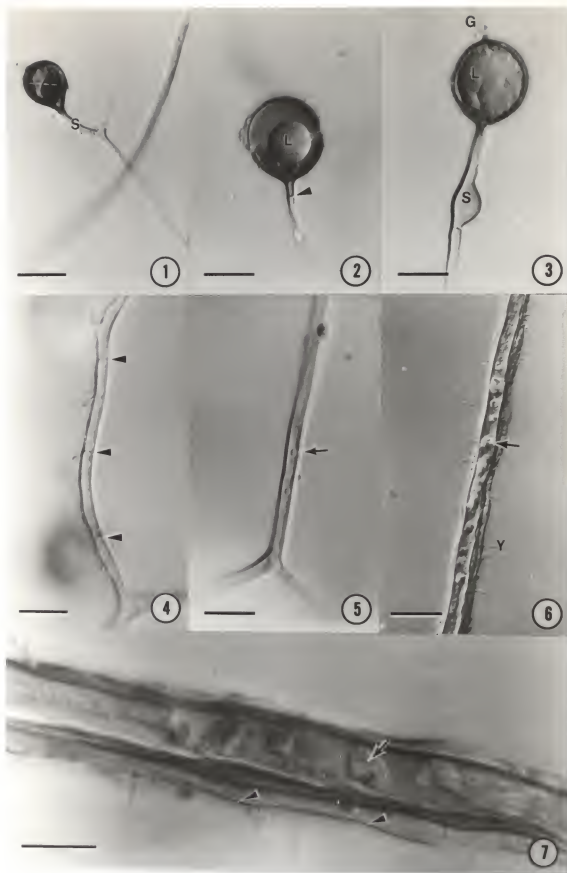
Figure 4.3 - General aspect of spore as seen with LM and  
Nomarsky interference contrast. Note germ tube  
(G), many lipid globules (L) inside the spore  
and subtending hypha with swelling (S). (Bar=  
20 $\mu$ m).

Figure 4.4 - Septate hypha (arrowheads). (Bar= 20  $\mu$ m).

Figure 4.5 - Coenocytic hypha with apparent nuclei (arrow).  
(Bar= 20  $\mu$ m).

Figure 4.6 - Interhyphal growth observed under Nomarsky  
interference contrast showing many nuclei  
distributed along the hypha (arrow) and  
microorganisms (Y) attached at the outer hypha.  
(Bar= 20  $\mu$ m).

Figure 4.7 - Detail of interhyphal growth (arrowheads) and  
nuclei (arrow). (Bar= 20  $\mu$ m).



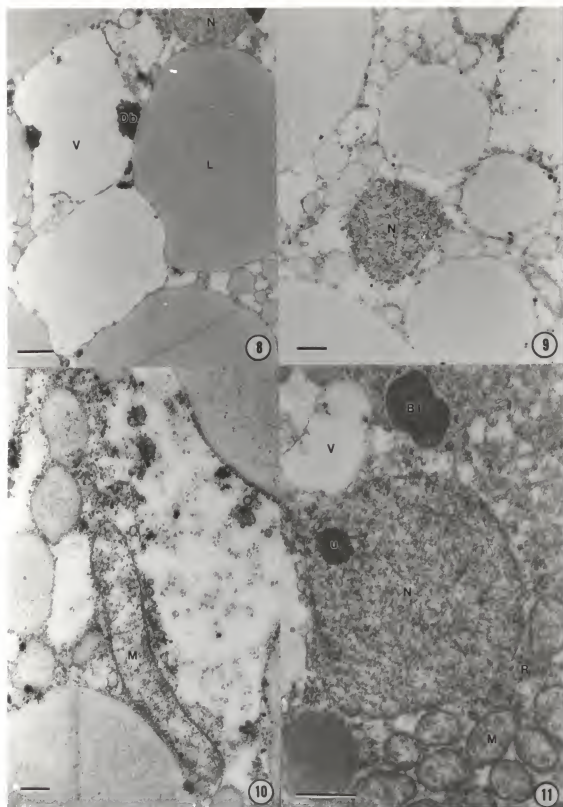
Figs. 4.8 - 4.11: Transmission electron micrographs of spores of *Glomus* sp. (L...925).

Figure 4.8 - General view of sporoplasm showing vacuoles (V), dense bodies (Db), lipid globule (L), and nucleus (N). (Bar= 1  $\mu$ m).

Figure 4.9 - Aspect of sporoplasm with small nucleus (N). (Bar= 0.5  $\mu$ m).

Figure 4.10 - Detail of sporoplasm at higher magnification showing mitochondria (M) and other sporoplasmic organelles. (Bar= 0.2  $\mu$ m).

Figure 4.11 - Fraction of sporoplasm with large nucleus (N) and evident nucleolus (u), ribosomes (R), mitochondria (M), vacuoles (V), and bacteria like organelle (Bl). (Bar= 0.5  $\mu$ m).



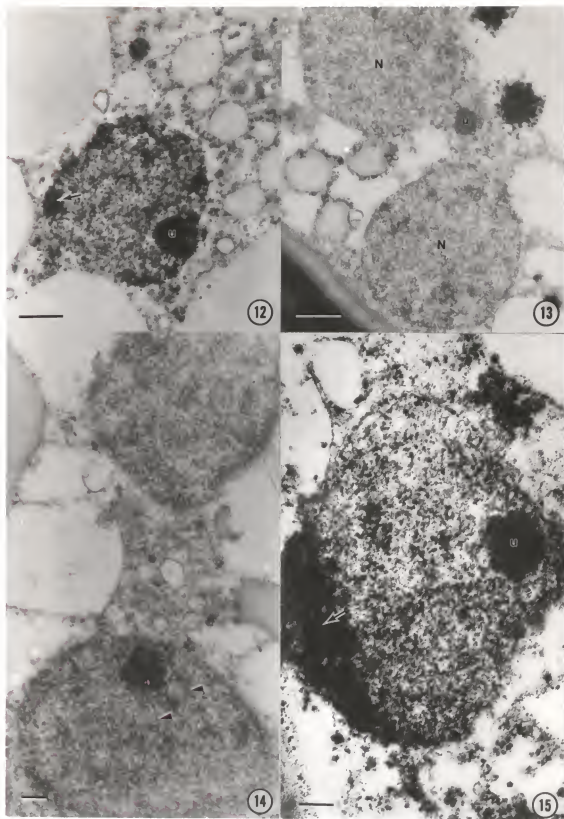
Figs. 4.12 - 4.15: Transmission electron micrographs of nuclei in spores of *Glomus* sp. (L...925).

Figure 4.12 - Nucleus with laterally localized nucleolus (u) and groups of condensed chromatin (arrow). (Bar= 0.5  $\mu$ m).

Figure 4.13 - Pair of nuclei (N) apparently connected by granular material. Note nucleolus (u) outside the nuclei and localized in the "bridge" which is connecting the two nuclei. (Bar= 0.5  $\mu$ m).

Figure 4.14 - Pair of nuclei. Note apparent microtubules (arrowhead). (Bar= 0.2  $\mu$ m).

Figure 4.15 - Detail of nuclei with lateral nucleolus (u) and condensed chromatin (arrow). (Bar= 0.2  $\mu$ m).



Figs. 4.16 - 4.20: Light and transmission electron micrographs of *Glomus intraradices* (LITR 208) spores.

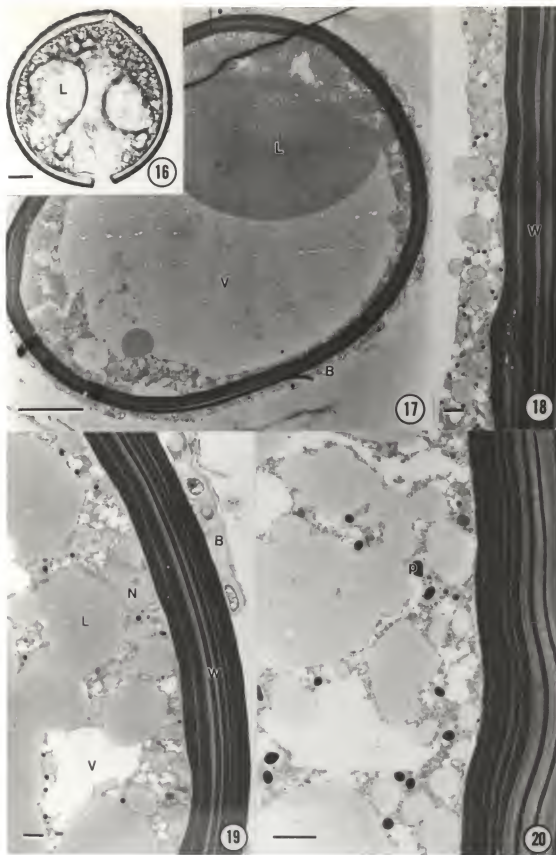
Figure 4.16 - General view of spore at the LM level. Note wall in one group (a) and lipid globules (L). (Bar= 20  $\mu\text{m}$ ).

Figure 4.17 - Aspect of spore as seen with TEM. Note large lipid globule (L), large vacuoles (V) and "layer" of bacteria (B) in the outer wall. (Bar= 1  $\mu\text{m}$ ).

Figure 4.18 - Distribution of organelles along the spore wall. Note laminated wall (W). (Bar= 1  $\mu\text{m}$ ).

Figure 4.19 - Segment of spore showing bacteria (B) in the outer wall, laminated wall and cytoplasmic organelles distributed between lipid globules (L), vacuoles (V) and the spore wall (W). Note one nucleus (N). (Bar= 1  $\mu\text{m}$ ).

Figure 4.20 - Detail of sporoplasm with many lipid globules, vacuoles and polyphosphate granules (p). (Bar= 1  $\mu\text{m}$ ).



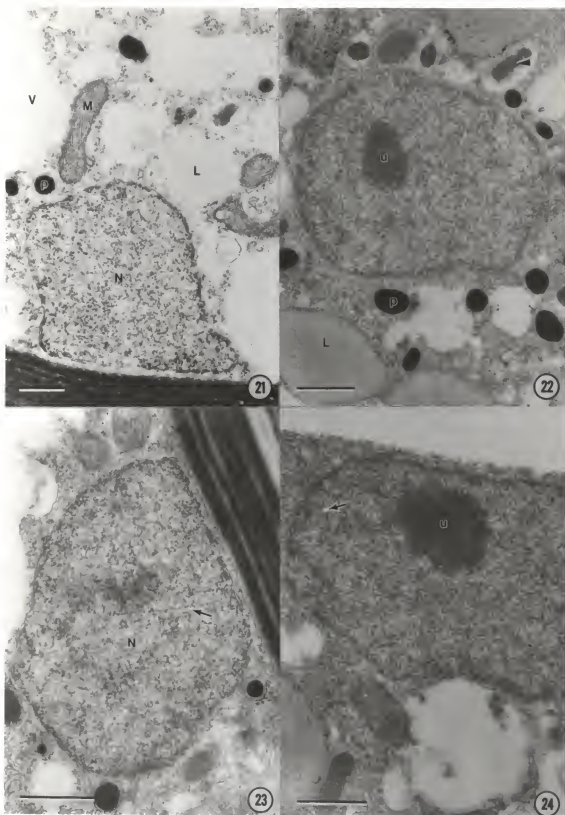
Figs. 4.21 - 4.24: Transmission electron micrographs of sporoplasm of *Glomus intraradices* (LITR 208).

Figure 4.21 - Detail of sporoplasmic organelles: nuclei (N), polyphosphate granules (p), mitochondria (M), vacuoles (V) and lipid globules (L). Note that nucleolus is not apparent in the nucleus. (Bar= 0.5  $\mu$ m).

Figure 4.22 - Aspect of sporoplasm showing large nuclei with evident nucleolus (u), vacuoles with and without content (arrowhead), polyphosphate granules (p), and lipid globules (L). (Bar= 0.5  $\mu$ m).

Figure 4.23 - Detail of nuclei (N) located near the spore wall. Note the lines of apparently microtubules (arrow) from one side to the center of the nucleus. (Bar= 0.5  $\mu$ m).

Figure 4.24 - A nucleus with a large nucleolus (u) and lines of apparently microtubules (arrow) starting from one side of the nucleus. (Bar= 0.5  $\mu$ m).



Figs. 4.25 - 4.30: Light and transmission electron micrographs of spores of *Glomus* sp. (L...906).

Figure 4.25 - General view of spore with LM. Note wall in one group (a). (Bar= 20  $\mu$ m).

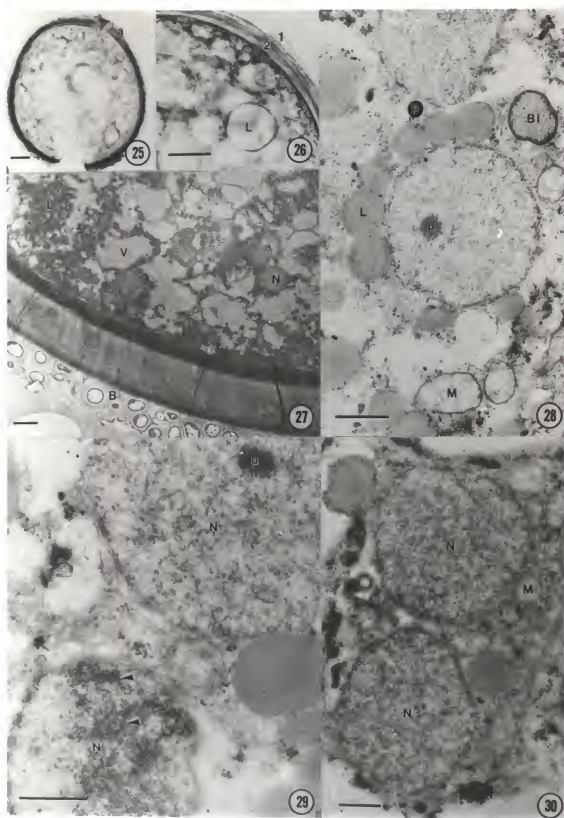
Figure 4.26 - Segment of spore showing walls (1, 2) and lipid globules (L). (Bar= 20  $\mu$ m).

Figure 4.27 - General aspect of spore at the TEM level. Note layer of bacteria (B) in the outer wall, nuclei (N), vacuoles (V), and group of lipid globules (L) in the sporoplasm. (Bar= 1  $\mu$ m).

Figure 4.28 - Detail of sporoplasm with mitochondria (M), nuclei with apparent nucleolus (u), bacteria like organelles (Bl), lipid globules (L) and polyphosphate granules (p). (Bar= 0.5  $\mu$ m).

Figure 4.29 - Pair of nuclei (N), one with evident nucleolus (u), the other with groups of condensed chromatin (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 4.30 - Pair of nuclei and closely associated mitochondria (M). (Bar= 0.5  $\mu$ m).



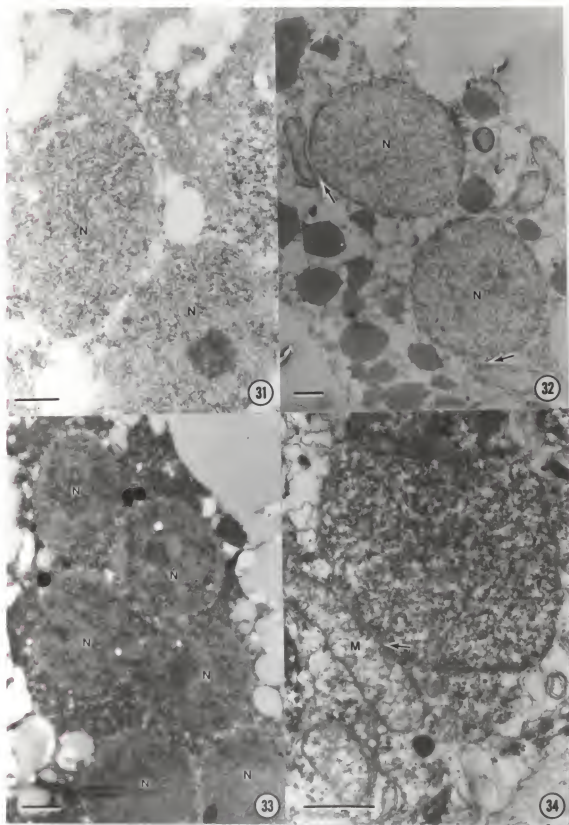
Figs. 4.31 - 4.34: Transmission electron micrographs of spores of *Glomus* sp. (L...906).

Figure 4.31 - Pair of nuclei (N). (Bar= 0.5  $\mu$ m).

Figure 4.32 - Pair of nuclei (N). Note connection of nuclear envelope and mitochondrial membrane (arrow). (Bar= 0.5  $\mu$ m).

Figure 4.33 - Group of six nuclei (N). (Bar= 0.5  $\mu$ m).

Figure 4.34 - Nucleus in close association (arrow) with mitochondria (M). (Bar=0.5  $\mu$ m).



Figs. 4.35 - 4.38: Transmission electron micrographs of germ tubes of *Glomus* sp. (L...906).

Figure 4.35 - General view of germ tube showing nucleus (N), vacuoles (V), and dense bodies (Db). (Bar= 1  $\mu$ m).

Figure 4.36 - Aspect of germ tube with nuclei and evident nucleolus (u), and vacuoles with dense bodies (Db). Note incorporation of new wall material (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 4.37 - Segment of germ tube with elongated mitochondria (M) and long microtubules (arrow). (Bar= 0.5  $\mu$ m).

Figure 4.38 - Higher magnification of mitochondria (M) and microtubules (arrow). (Bar= 0.5  $\mu$ m).



Figs. 4.39 - 4.44: Transmission electron micrographs of germ tubes of *Glomus* sp. (L...906).

Figure 4.39 - Segment of hypha showing lomasomes (Lo), vacuoles (V) and dense bodies (Db). (Bar= 0.5  $\mu$ m).

Figure 4.40 - Detail of hypha with apparently empty vacuoles (V) and vacuoles filled with dense bodies (Db). (Bar= 0.5  $\mu$ m).

Figure 4.41 - Portion of hypha showing large vacuoles (V) including small vacuoles with double membranes (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 4.42 - Bacteria like organelle (BLO): beginning of fission (arrowhead). (Bar= 0.2  $\mu$ m).

Figure 4.43 - Advanced phase of binary fission in a BLO (arrowhead). (Bar= 0.2  $\mu$ m).

Figure 4.44 - More advanced stage of division in a BLO (arrowhead). (Bar= 0.2  $\mu$ m).

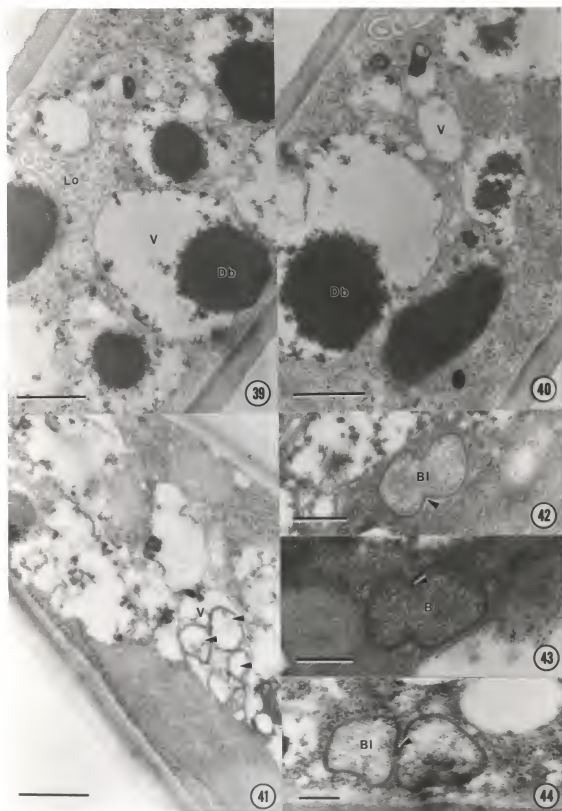


Figure 4.45 - 4.51: Light and transmission electron micrographs of spores and hypha of *Gigaspora albida* (GABD 927).

Figure 4.45 - Light microscope micrograph of spore with several germ tubes (G) developing near the sporogenous cell (arrowhead). (Bar= 50  $\mu$ m).

Figure 4.46 - Detail of spore and germ tubes with LM. Note sporogenous cell (S). (Bar= 50  $\mu$ m).

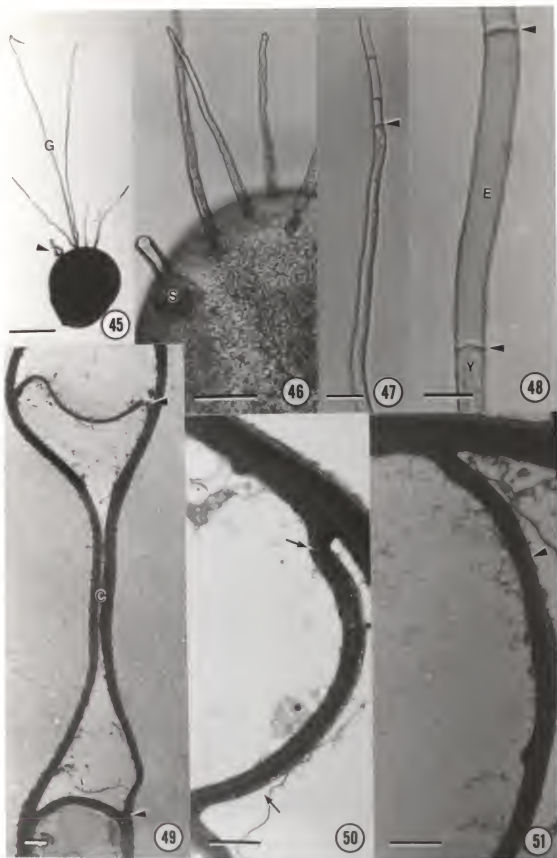
Figure 4.47 - Apex of germ tube with septa (arrowhead) and empty segments. (Bar= 20  $\mu$ m).

Figure 4.48 - Detail of germ tube showing empty segments (E) separated by septa (arrowhead) from living segment (Y). (Bar= 5  $\mu$ m).

Figure 4.49 - Septate germ tube (arrowhead) with collapsed segment (C). (Bar= 1  $\mu$ m).

Figure 4.50 - Detail of septum with plasmodesmata not very clear (arrows). (Bar = 5  $\mu$ m).

Figure 4.51 - Detail of septum (arrowhead) with plasmodesmata not evident. (Bar= 0.5  $\mu$ m).



Figs. 4.52 - 4.58: Light, scanning and transmission electron micrographs of spores of *Gigaspora albida* (GABD 927).

Figure 4.52 - Detail of spore at SEM level showing sporogenous cell (S) and two germ tubes (G). (Bar= 20  $\mu\text{m}$ ).

Figure 4.53 - Higher magnification of sporogenous cell (S) with SEM. Note hyphal segment (H) originating from the sporogenous cell. (Bar= 10  $\mu\text{m}$ ).

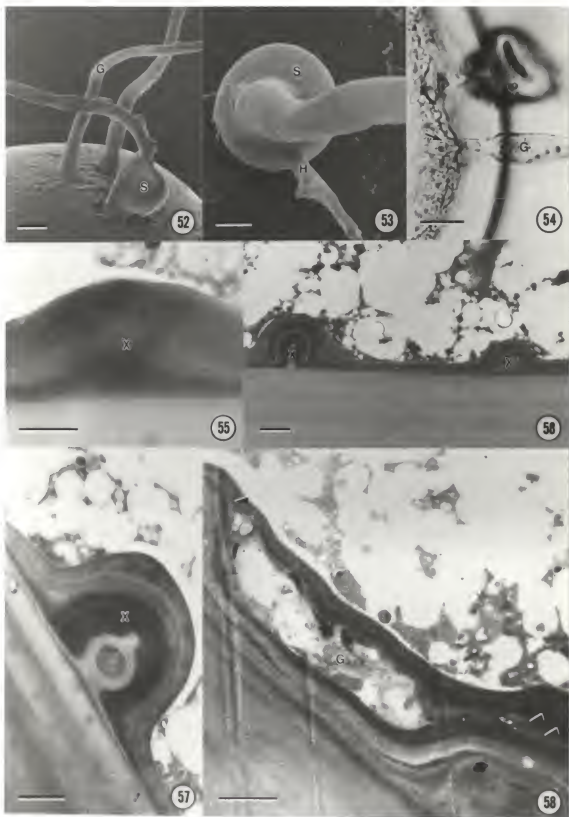
Figure 4.54 - Detail of germ tube (G) emerging from the spore at the LM level. Note that there is a wall separating its base from the sporoplasm (arrow). (Bar= 20  $\mu\text{m}$ ).

Figure 4.55 - Formation of wall protuberance or projection (X). (Bar= 0.5  $\mu\text{m}$ ).

Figure 4.56 - General view of spore with two projections (X) which are being formed by the innermost wall. (Bar= 1  $\mu\text{m}$ ).

Figure 4.57 - Detail of spore wall protuberance (X) formed by material of different electron density (Bar= 1  $\mu\text{m}$ ).

Figure 4.58 - Early stage of germ tube (G) formation. Note that wall layers of wall four are separating to allow development of the germ tube (arrowhead). (Bar= 1  $\mu\text{m}$ ).



Figs. 4.59 - 4.63: Transmission electron micrographs showing different phases of germ tube development in *Gigaspora albida* (GABD 927).

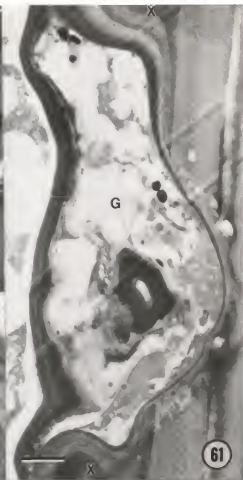
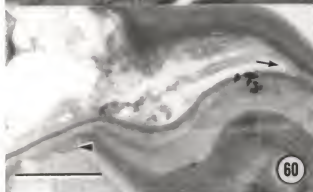
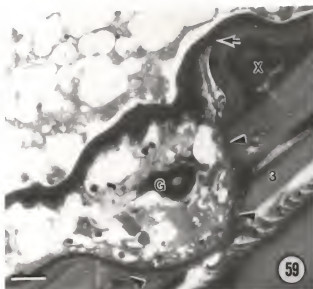
Figure 4.59 - Early phase of germ tube (G) formation showing degradation of the third wall (3), formation "de novo" of a wall (arrowhead), and separation of layers of wall four (arrow) to allow development of germ tube. Note that separation starts on the projection (X) of the wall. (Bar= 1  $\mu$ m).

Figure 4.60 - Detail of region where layers of wall four start separating (arrow) for development of germ tube. Note "de novo" formation of wall around the germ tube (arrowhead). (Bar= 1  $\mu$ m).

Figure 4.61 - General view of germ tube (G) in a more advanced stage. Note that it is localized between two projections of the wall (X). (Bar= 1  $\mu$ m).

Figure 4.62 - More advanced stage of germ tube initial (G) showing detail of wall three (3) partially degraded and new wall (arrowhead). (Bar = 1  $\mu$ m).

Figure 4.63 - Emergence of germ tube. Observe differentiation of two wall layers (1, 2) and high vacuolization (V) of the germ tube. (Bar= 1  $\mu$ m).



Figs. 4.64 - 4.69: Transmission electron micrographs of developed germ tubes of *Gigaspora albida* (GABD 927).

Figure 4.64 - General view of hypha showing distribution of nuclei (N) and other cytoplasmic organelles. (Bar= 1 $\mu$ m).

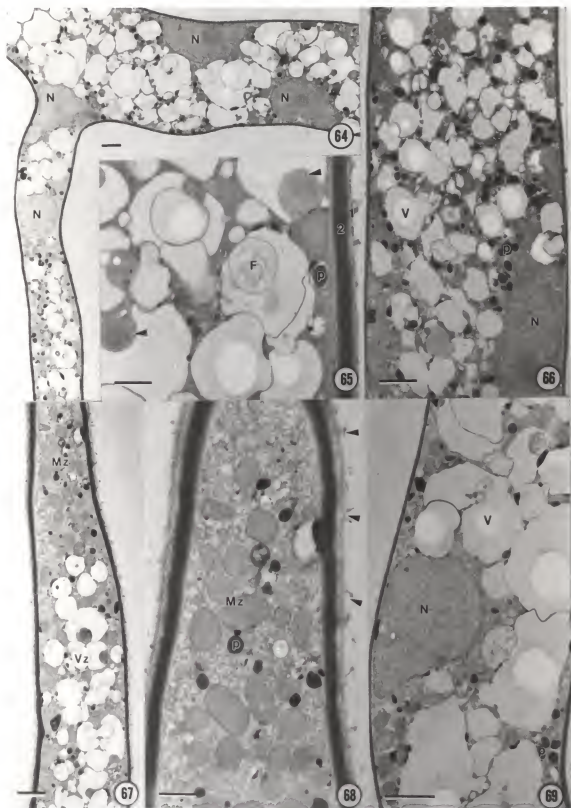
Figure 4.65 - Detail of hyphal segment showing vacuoles with apparent myelin figures (F), vacuoles enclosing cytoplasmic material (arrowhead) and polyphosphate granules (p). Note wall with two defined layers (1, 2). (Bar= 0.5  $\mu$ m).

Figure 4.66 - General aspect of hyphal segment with a nucleus (N), many vacuoles (V) and polyphosphate granules (p) dispersed throughout the cytoplasm. (Bar= 1  $\mu$ m).

Figure 4.67 - Segment of hypha showing two distinct zones: a mitochondrial zone (Mz) and a vacuolation zone (Vz). (Bar= 1  $\mu$ m).

Figure 4.68 - Detail of mitochondrial zone (Mz) in the hypha which shows also many polyphosphate granules (p). Note outer wall which is sloughing (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 4.69 - Aspect of hyphal segment with a nucleus (N) localized near the wall, many vacuoles (V) and polyphosphate granules (p). (Bar= 1  $\mu$ m).



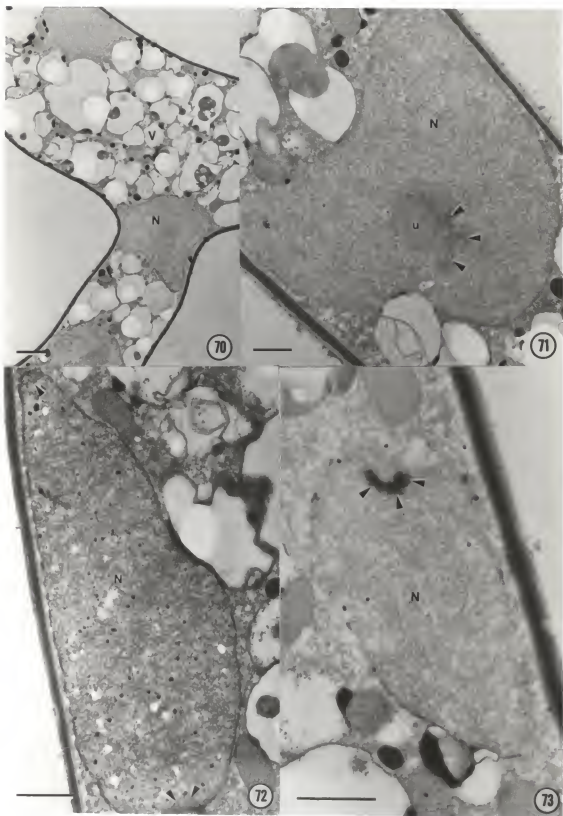
Figs. 4.70 - 4.73: Transmission electron micrographs of hypha of *Gigaspora albida* (GABD 927).

Figure 4.70 - General view of hyphal segment with nuclei (N) and several vacuoles (V). (Bar= 1  $\mu$ m).

Figure 4.71 - Detail of large nucleus (N) occupying all hyphal width. Note nucleolus (u) and unidentified material (arrowhead) inside the nucleus. (Bar= 1  $\mu$ m).

Figure 4.72 - High magnification of elongate nucleus which is located near the wall and shows electron dense, not identified material (arrowhead) near its poles. (Bar= 0.5  $\mu$ m).

Figure 4. 73 - Detail of nucleus (N) with electron dense band (arrowhead) adjacent to the nuclear membrane, which could be the nucleus associated organelles (= NAOs). (Bar= 0.5  $\mu$ m).



Figs. 4.74 - 4.77: Transmission electron micrographs of hypha of *Gigaspora albida* (GABD 927).

Figure 4.74 - High magnification of elongate nucleus (N). (Bar= 0.5  $\mu$ m).

Figure 4.75 - Detail of nucleus with microtubules (arrowhead) and condensed chromatin (c). (Bar= 0.5  $\mu$ m).

Figure 4.76 - General aspect of hypha with a nucleus localized near the wall. Note possible NAOs adjacent to the nuclear envelope (arrowhead). (Bar= 0.5  $\mu$ m).

Figure 4.77 - Detail of a nucleus (N) which seems to be dividing in the central region (arrow). Note lines of microtubules from one pole to the other nuclear pole (arrowhead). (Bar= 0.5  $\mu$ m).



## CHAPTER V SUMMARY AND CONCLUSIONS

Separated in a single order of Zygomycetes, Glomales, the arbuscular mycorrhiza-forming fungi are a group with very peculiar and unique characteristics such as development of arbuscules and the obligate mutualistic symbiotic association with plant roots. Although the subject of many studies, they constitute the most poorly understood group of Zygomycetes. Part of these difficulties are related to the reproductive structures they produce, which have consistently been a matter of discussion. In fact, there is no clear evidence of the sexual or asexual nature of the spores formed by the arbuscular mycorrhizal fungi, although attempts have been made repeatedly to overcome this problem. Cytological studies would provide information on nuclear condition of the spores, thereby answering this most important question about the group, and providing a better understanding of Glomales. However, there are only a few cytological studies on Glomales. This is the result of difficulties related to fixation and embedding of spores which, being thick walled, cannot be easily penetrated by fixatives and embedding resins. Thus, one of the first objectives of this work was to develop fixation and

embedding techniques which would provide reasonable material for cytological studies.

It was observed that for walls, most of the nine methods tested can be considered satisfactory; however, for the sporoplasm, very few techniques provided feasible fixation and embedding with the results varying greatly among the isolates that were studied. With some restrictions, the only technique which supplied satisfactory results for *Glomus* isolates was the one in which the spores were fixed with glutaraldehyde in a microwave oven, and broken in liquid N<sub>2</sub>, before postfixation with osmium tetroxide. However, the method did not work well for species of *Gigaspora*, *Acaulospora* or *Scutellospora*. This indicates that it will be necessary to develop different procedures for fixation and embedding of each genera, or even species, of arbuscular mycorrhizal fungi since the response to the tested techniques has been quite different among the taxa.

Spore walls have been widely used as a diagnostic feature for identification of species of Glomales since they were proved to be useful taxonomic characters. Ultra-structural examinations confirmed that *Glomus* and *Gigaspora* have spore walls in one group while spore walls of *Acaulospora* species are distributed in two or more groups. Presence of a wall with apparent bow-shaped arrangement of fibrils was quite common among isolates of the different

genera examined. It indicates that is an ordinal rather than a generic or familial characteristic. Moreover, the apparent arced distribution of fibrils cannot be thought of as a peculiarity of laminated walls since those walls were shown to have arched and other ultrastructural fibrillar arrangements. In the same way, walls with this fibrillar arrangement are not always seen as laminated when observed at the LM level. Different developmental processes were associated with formation of laminated walls as exemplified here with *Glomus intraradices* and *Gigaspora albida*. A special feature detected in the wall of *Acaulospora scrobiculata* (ASCB 984) was a perpendicular periodic layer where fibrils apparently have a radial arrangement. This has been shown in other genera of arbuscular mycorrhizal fungi, such as *Acaulospora laevis* and *Scutellospora nigra*. This might be a general characteristic of spores with more than one wall group which could be confirmed by further studies with other Glomales species. The striate layer reported in walls of *Gigaspora margarita* and in *Scutellospora gregaria* was also found here in spores of *Gigaspora albida*. It may be possible that this constitutes a familial characteristic.

Bacteria have been reported as habitual colonizers of spores of arbuscular mycorrhizal fungi. It was observed in this study that the bacteria degrade only the outer wall of spores which are known to have an outer, evanescent wall.

Therefore, these organisms could be an important factor in the process of degradation of these walls. It may be possible that the function of the evanescent wall is to protect the spore integrity until a permanent, more resistant wall has been fully developed. In spores with only permanent walls, such as *Acaulospora scrobiculata*, bacteria were present in the hollows of the ornamented wall without, apparently, degrading that wall. This seems to indicate that some constituent of the permanent wall is not susceptible to the enzymatic degradation produced by the bacteria. In some *Glomus* spores, an electron dense layer separated the outer, evanescent wall from the inner, permanent wall(s). This layer has been reported to be formed by sporopollenin and it is probable that in these cases this layer, being highly resistant, constitutes a barrier for the bacteria and maintain the integrity of the spores.

The characters used for generic classification of Glomales have been somatic and reproductive structures, spore ontogeny, and the types of spore germination. Ontogeny of spores are visibly different in Glomaceae, Acaulosporaceae and Gigasporaceae, and within their genera. Spore germination, however, has not been clearly distinguished in some cases, because very few detailed studies have been done in this area. A different type of germination was observed to occur in *Gigaspora albida*. The

process includes formation of a germination chamber, originated between the inner spore walls, and from which the germ tube initial arises. This special area develops between rounded projections of the innermost wall. Such projections were earlier defined as characteristics of the germinal wall. This type of germination seems to be an intermediary form between the germination processes described for *Gigaspora margarita*, where the germ tube simply emerges through the wall, and for species of *Scutellospora*, where there is development of germ shields. In fact the process described here seems slightly similar to the germination of *Acaulospora laevis* spores, with the difference that peripheral compartments have not been formed. *Acaulospora* and *Gigaspora* are very distinct genera which share few characteristics in common, especially regarding spore ontogeny and wall structure. Consequently to find a slightly similar form of germination between them was not expected. It might be suggested, however, that this form of germination is a character which arose independently in each group.

A special feature observed in some spores was the connection of the outer mitochondrial membrane with the nuclear envelope, which had not been reported in other arbuscular mycorrhizal fungi. The significance of this connection, however, is not known.

Although no clear evidence of mitosis or meiosis in the nuclei of spores and germ tubes was found, it is possible to conclude that nuclear processes related with division were occurring. In several instances, for example, faintly stained microtubules were observed, and undescribed structures which appear to be nucleus associated organelles (NAOs) were seen. On one occasion, paired nuclei were linked, but unfortunately it could not be determined if they were in the process of division or fusion. In another case, a defined line was seen dividing a nucleus in its central region. Lines corresponding to microtubules crossed the same nucleus from one pole to the other. Poor fixation and staining were the main reasons why these processes were not clearly seen and defined. An abundant number of paired spore nuclei, the elongation and blebbing of others are indicative of nuclear fusion or division. One hypothesis to be considered is that the nuclei could perhaps fuse in the spore and later in the germ tube they would start to divide first meiotically and later mitotically, in that way providing the mycelium with enough nuclear material for expansion. But there is also the possibility that they divide only mitotically without any nuclear fusion. Clearly further cytological and developmental studies are necessary to clarify the formation and nuclear condition of spores in each genera of Glomales. It could not be determined if

these spores are chlamydospores, sporangiospores, conidia, azygospores or zygosporos.

The characteristics of the order Glomales seems to indicate that they are correctly placed among the Zygomycetes. Many of the wall constituents known in Zygomycetes, including sporopollenin, were also reported to occur in Glomales. Septal perforations, as found in *Glomus*, *Sclerocystis* and now in *Gigaspora*, are also present in Endogonales and Mucorales, thus associating these groups in a same class. Another character linking Glomales to Zygomycetes is the "de novo" formation of a new wall, which was shown here to occur during spore germination of *Gigaspora albida*. Mucorales are considered to have the most primitive type of eukaryotic division. The fact that division processes were not seen clearly in Glomales may indicate that they have a very primitive type of division in which its features are difficult to characterize. The features observed in some Glomales nuclei may indicate that they also have a closed, acentric mitosis, with absence of recognizable chromosomes, persistent nucleolus, and NAO's associated with the inner nuclear membrane. This is in agreement with the division process of most Zygomycetes.

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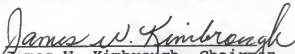
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## BIOGRAPHICAL SKETCH

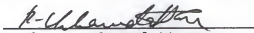
Leonor Costa Maia was born in Recife, Pernambuco, Brazil, where she grew up and got her education. She finished her high school studies in 1968 and obtained the degree of Bachelor in Biological Sciences at the Faculdade de Filosofia do Recife/Universidade Federal de Pernambuco (UFPE) in 1972. In 1970 she started her training in mycology at the Departamento de Micologia/UFPE. From 1974 to 1977 she was a botany and ecology college teacher at the Faculdade de Filosofia do Recife, and in 1977 she started working at the Departamento de Micologia/UFPE. She initiated her graduate studies at that time and earned a Master of Science degree in botany at the Universidade Federal Rural de Pernambuco (UFRPE) in 1980. She continued working at the Departamento de Micologia as an auxiliary professor and subsequently as assistant professor. After initiating research in mycorrhizae, she decided to apply for a scholarship in order to undertake a Ph.D. program in this area. In 1987, she obtained a study leave and entered a graduate program in the Department of Plant Pathology at the University of Florida. After concluding her Ph.D., she plans to reassume her position as adjunct professor at the Universidade Federal de Pernambuco. She is a member of the

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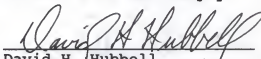
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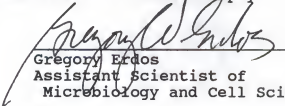
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